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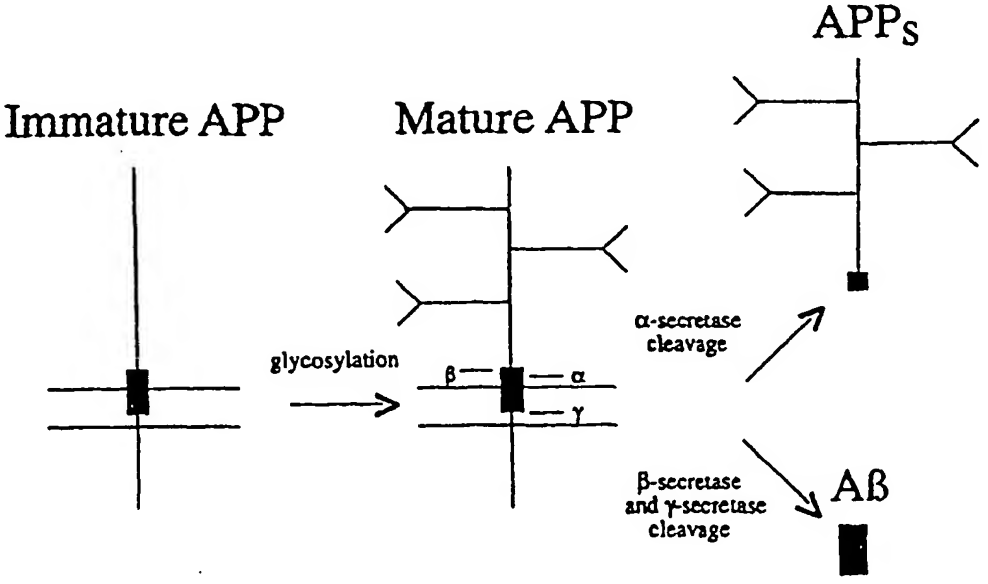
(54) Title: METHOD OF TREATING AMYLOID β PRECURSOR DISORDERS



(57) Abstract: Methods for the treatment and prevention of APP processing disorders such as Alzheimer's disease and Down's Syndrome which are based on the administration of an effective amount of a HMG-CoA reductase inhibitor to a mammal are disclosed. Additionally, methods for the treatment and prevention of APP processing disorders such as Alzheimer's disease and Down's Syndrome which are based on the reduction of cellular cholesterol in a mammal are disclosed. These methods reduce the amount of Aβ peptides or decrease the formation of Aβ peptides or increase the clearance of Aβ peptides in a mammal suffering from Alzheimer's disease and Down's Syndrome.

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METHOD OF TREATING AMYLOID β PRECURSOR DISORDERS

RELATED APPLICATION DATA

This application claims the benefit of U.S. Provisional Patent Application No. 60/163,608, filed November 4, 1999, U.S. Provisional Patent Application No. 60/219,435, filed July 22, 2000, and U.S. Provisional Patent Application No. 60/223,987, filed August 9, 2000, naming Drs. Lawrence Friedhoff and Joseph Buxbaum as co-inventors. The aforementioned applications are herein incorporated by reference.

TECHNICAL FIELD

The present invention relates to a method of treating amyloid β precursor protein (APP) disorders such as Alzheimer's disease and Down's Syndrome.

BACKGROUND OF THE INVENTION

The cause of Alzheimer's disease is not known. The disease is characterized by the accumulation of β-amyloid peptides (Aβ peptides), as abnormal protein precipitates, in the brain. It is generally believed that these proteins kill brain cells which causes a loss of mental function.

As illustrated in Figure 1, immature amyloid β precursor protein (APP_i) under-goes glycosylation to become mature amyloid β precursor protein (APP_m). Then APP_m is either (1) cleaved by the protease α-secretase to produce a secreted form of APP (APP_s) which is not amyloidogenic, or (2) cleaved by β-secretase and γ-secretase to produce the abnormal protein, Aβ (Aβ peptide), which can then precipitate.

Many advances have been made in the treatment of Alzheimer's disease. The cholinesterase inhibitors such as tacrine, donepezil and rivastigmine improve

symptoms slightly. However, the slight improvement in attention and alertness is most likely due to increased brain acetylcholine levels. Unfortunately, however, the cholinesterase inhibitors do not prevent cognitive decline, which is inevitably fatal even with optimal cholinesterase inhibitor treatment.

Several strategies for treating Alzheimer's disease have been proposed and include decreasing or preventing the release of Aβ peptide by either increasing α-secretase or decreasing the β- or γ-secretase activity or production. Other strategies include decreasing Aβ peptide aggregation, increasing Aβ peptide clearance, reducing Aβ peptide production or decreasing the cellular effects of Aβ peptide aggregation and deposition. *See Sabbagh, M. N. et al.,* (1997) Alzheimer's Disease Rev. 3:1-19. See also U.S. Patent No. 6,080,778. In light of the foregoing, there is a need for a more effective treatment of mammals suffering from APP processing disorders such as Alzheimer's disease and Down's Syndrome.

SUMMARY OF THE INVENTION

Generally, the invention relates to a method for treating a mammal having an APP processing disorder comprising administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor. APP processing disorders include Alzheimer's disease and Down's Syndrome.

In a preferred embodiment, the invention relates to a method of treating a mammal having Alzheimer's disease and/or Down's Syndrome by administering to the mammal a therapeutically effective amount of at least one HMG-CoA reductase inhibitor. In this embodiment, the method may also comprises determining whether

the mammal exhibits at least one objective symptom of Alzheimer's disease or Down's Syndrome.

In another embodiment of the present invention, the composition comprising at least one HMG-CoA reductase inhibitor may further comprise a pharmaceutically acceptable excipient. The composition is preferably in the form of a controlled release formulation.

In a preferred embodiment of the present invention, the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, lovastatin, rivastatin and fluvastatin, and pharmaceutically effective salts, isomers and the active metabolite forms thereof, or a combination thereof. In a more preferred embodiment, the HMG-CoA reductase inhibitor is lovastatin or lovastatin acid.

In another preferred embodiment, about 0.2 mg to about 10 mg of the HMG-CoA reductase inhibitor per Kg of the mammal's body weight per day is administered. The daily amount administered to the mammal may be administered in more than one fraction.

In another preferred embodiment, an oral dose of about 5 mg to about 400 mg of lovastatin per day is administered to a human having an APP processing disorder. In a more preferred embodiment the oral dose is about 10 mg to about 350 mg per day. More preferably, the oral dose is about 10 mg to about 300 mg per day. Even more preferably, the oral dose is about 10 mg to about 250 mg per day.

In another preferred embodiment, any suitable dose of an HMG-CoA reductase inhibitor is administered to a mammal having an APP processing disorder. More preferably, the suitable dose is one that is therapeutically effective and results in the

average blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state being below about 50 micromolar. More preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 30 micromolar. Even more preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 20 micromolar. In an even more preferred embodiment, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 10 micromolar. Even more preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 5 micromolar. Even more preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 1 micromolar. Most preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is about 0.5 micromolar.

In another embodiment, the invention relates to a method for treating a mammal having an APP processing disorder which comprises lowering the amount of Aβ peptides in the brain, cerebral spinal fluid, or plasma of the mammal by administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor. Lowering the amount of Aβ peptides in the brain may comprise affecting APP_m processing. In a preferred embodiment, the amount of Aβ peptides is lowered in the brain of the mammal.

In another embodiment, the invention relates to a method for treating a mammal having an APP processing disorder which comprises increasing the clearance of Aβ peptides in the brain, cerebral spinal fluid, or plasma of the mammal by

administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor. In a preferred embodiment, the clearance of Aβ peptides in the brain of the mammal is increased.

In another embodiment, the invention relates to a method for treating a mammal having an APP processing disorder comprising preventing or reducing Aβ peptide aggregation or plaque formation in the brain of the mammal by administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

In another embodiment, the invention relates to a method for the treatment of a mammal exhibiting the objective symptoms of Alzheimer's disease by decreasing the formation of Aβ peptides, increasing the clearance of Aβ peptides, regulating the processing of APP, or reducing plaque maturation in the mammal by administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

In another embodiment, the invention relates to a method for treating a mammal having an APP processing disorder comprising lowering the amount cellular cholesterol levels in the mammal. In a preferred embodiment, the amount of cellular cholesterol levels are decreased by the administration of at least one HMG-CoA reductase inhibitor.

Generally an immediate release or a controlled release dosage form may be utilized in the practice of the invention. The immediate release dosage formulation may comprise an effective amount of a HMG-CoA reductase inhibitor and a suitable pharmaceutical diluent. The controlled release dosage formulation may comprise:

a compressed tablet core which contains an alkyl ester of a hydroxy substituted naphthalene derivative, a pharmaceutically acceptable, water swellable polymer and an osmotic agent; and

an outer coating layer which covers the osmotic core and comprises a pH sensitive coating agent and a water insoluble polymer.

An optional sealing coat may be applied to the compressed tablet core and an optional coating layer comprising an enteric coating agent may be applied under the outer coating layer as an inner coating or as an overcoat over the outer coating layer. The tablet core may be compressed using a smooth faced tablet die. The preferred alkyl ester of a hydroxy substituted naphthalene compound is lovastatin. Plasma levels of about 0.5 micromoles of the HMG-CoA reductase inhibitor are preferably maintained by the use of a controlled release formulation of the HMG-CoA reductase inhibitor.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic which illustrates APP processing.

Figures 2a and 2b illustrate the effects of lovastatin acid on Aβ peptides in Human Neuroglioma (H4) cells. Figure 2a is a photograph of two gel wells wherein the negative well and the positive well correspond to the bar graphs of Figure 2b representing 0 and 0.5 μM of lovastatin acid, respectively. Data represent the mean ± the standard error of the mean (SEM) of one experiment performed in quadruplicate.

Figures 3a and 3b illustrate the effects of lovastatin acid on Aβ peptides in Madin-Darby Canine Kidney (MDCK) cells. Figure 3a is a photograph of two gel wells wherein the negative well and the positive well correspond to the bar graphs of

Figure 3b representing 0 and 0.5 μM of lovastatin acid, respectively. Data represent the mean ± SEM of three experiments performed in quadruplicate.

Figures 4a and 4b illustrate the effects of lovastatin acid on Aβ peptides in Chinese Hamster Ovary (CHO) cells. Figure 4a is a photograph of two gel wells wherein the negative well and the positive well correspond to the bar graphs of Figure 4b representing 0 and 0.5 μM of lovastatin acid, respectively. Data represent the mean ± SEM of four experiments performed in quadruplicate.

Figures 5 illustrates the effects of lovastatin acid on APP_s processing. Data represent the mean ± SEM of an experiment performed in quadruplicate.

Figure 6 illustrates the effects of lovastatin acid on mature APP processing. Data represent the mean ± SEM of an experiment performed in quadruplicate.

Figure 7 is a graph showing the steady-state plasma concentrations of lovastatin acid in patients after multiple oral 40 mg doses of Lovastatin XL, a preferred extended release tablet form of lovastatin.

Figure 8 is a graph showing the change in the mean Aβ peptide concentration in the blood of groups of patients after treatment with various doses of Lovastatin XL.

Figure 9 is a bar chart showing the change in the mean Aβ peptide concentration in the blood of groups of patients after treatment with various doses of Lovastatin XL.

DETAILED DESCRIPTION OF THE INVENTION

Recently, the present inventors have discovered that HMG-CoA reductase inhibitors lower the amount of Aβ peptide levels, prevent or reduce Aβ peptide formation, may increase Aβ clearance, and therefore prevent or reduce Aβ peptide aggregation. More particularly, the present inventors have discovered that the

administration of HMG-CoA reductase inhibitors lower the amount of Aβ peptide levels, prevent or reduce Aβ peptide formation, may increase Aβ clearance, and therefore prevent or reduce Aβ peptide aggregation, without the need of other cholesterol lowering treatments. Therefore, methods of treating APP processing disorders such as Alzheimer's disease and Down's Syndrome in a mammal comprising the administration of a HMG-CoA reductase inhibitor to the mammal is disclosed herein below.

As used herein, "APP_i" means the immature form of amyloid β protein precursor, "APP_m" means the mature form of amyloid β protein precursor, "APP_s" means the amyloid β protein precursor which is cleaved by α-secretase and which is the secreted form, "APP" means either APP_i, APP_m, or both.

As used herein, "post-translational" events include the cleavage of APP_m by β- and γ-secreatases.

As used herein, "other cholesterol lowering treatments" means any treatment other than treatment with a HMG-CoA reductase inhibitor. Other cholesterol lowering treatments include, but are not limited to, treatment with mevalonate, methyl-ß-cyclodextrin, and/or cyclodextrin.

As used herein, "active metabolite" is intended to mean a pharmacologically active product produced through metabolism in the body of a specified compound or salt thereof. Active metabolites of a compound may be identified using routine techniques known in the art. See, e.g., Bertolini, G. et al., *J. Med. Chem.*, 40, 2011-2016 (1997); Shan, D. et al., *J. Pharm. Sci.*, 86 (7), 765-767; Bagshawe K., *Drug Dev. Res.*, 34, 220-230 (1995); Bodor, N., *Advances in Drug Res.*, 13, 224-331 (1984); Bundgaard, H., *Design of Prodrugs* (Elsevier Press 1985); and Larsen, I. K., *Design*

and Application of Prodrugs, Drug Design and Development (Krogsgaard-Larsen et al., eds., Harwood Academic Publishers, 1991).

As used herein, the term "pharmaceutically acceptable salts" refers to salt forms that are pharmacologically acceptable and substantially non-toxic to the subject being administered the composition of the present invention. Pharmaceutically acceptable salts include conventional acid-addition salts or base-addition salts formed from suitable non-toxic organic or inorganic acids or inorganic bases. Exemplary acid-addition salts include those derived from inorganic acids such as hydrochloric acid, hydrobromic acid, hydroiodic acid, sulfuric acid, sulfamic acid, phosphoric acid, and nitric acid, and those derived from organic acids such as p-toluenesulfonic acid, methanesulfonic acid, ethane-disulfonic acid, isethionic acid, oxalic acid, p-bromophenylsulfonic acid, carbonic acid, succinic acid, citric acid, benzoic acid, 2-acetoxybenzoic acid, acetic acid, phenylacetic acid, propionic acid, glycolic acid, stearic acid, lactic acid, malic acid, tartaric acid, ascorbic acid, maleic acid, hydroxymaleic acid, glutamic acid, salicylic acid, sulfanilic acid, and fumaric acid. Exemplary base-addition salts include those derived from ammonium hydroxides (e.g., a quaternary ammonium hydroxide such as tetramethylammonium hydroxide), those derived from inorganic bases such as alkali or alkaline earth-metal (e.g., sodium, potassium, lithium, calcium, or magnesium) hydroxides, and those derived from organic bases such as amines, benzylamines, piperidines, and pyrrolidines.

Any HMG-CoA reductase inhibitor may be used in the method of the present invention. The term "HMG-CoA reductase inhibitor" refers to any one or more compounds that inhibit the bioconversion of hydroxymethylglutamyl-coenzyme A to mevalonic acid which is catalyzed by the enzyme HMG-CoA reductase. Such

inhibition may be determined by standard methods known to those of ordinary skill in the art. Examples of suitable HMG-CoA reductase inhibitors are described and referenced herein, however, other HMG-CoA reductase inhibitors will be known to those of ordinary skill in the art. Therefore, the present invention should not be limited to the specific HMG-CoA reductase inhibitors exemplified herein.

Examples of such HMG-CoA reductase inhibitors which are useful in the method of the present invention for the treatment of Alzheimer's disease include mevastatin which is described in U.S. Pat. No. 3,671,523; lovastatin which is described in U.S. Pat. No. 4,231,938; pravastatin which is described in U.S. Pat. No. 4,346,227; simvastatin which is described in U.S. Pat. No. 4,444,784; atorvastatin which is described in U.S. Pat. No. 4,647,576; rivastatin which is described in European Pat. No. 491226A; and fluvastatin which is described in U.S. Pat. No 4,739,073. All of these patents are incorporated herein by reference. Further, any suitable isomers of the exemplified HMG-CoA reductase inhibitors may be used, including stereoisomers, enantiomers, or mixtures thereof and, thus, their use in pharmaceutical formulations for the treatment of APP disorders are within the scope of the invention.

Lovastatin is a metabolite which is produced by the natural fermentation of a fungus of the Aspergillus genus. The other compounds of this class are derived from natural and synthetic sources using well known procedures and have similar mechanisms of activity.

Any suitable method for administering the HMG-CoA reductase inhibitor may be used. For example, the HMG-CoA reductase inhibitors may be administered orally

to a mammal having Alzheimer's disease or Down's Syndrome an effective amount to relieve the symptoms of Alzheimer's disease or Down's Syndrome.

Preferably, the effective amount of the HMG-CoA reductase inhibitor results in the average blood plasma concentrations of the HMG-CoA reductase inhibitor or its active metabolite at steady-state being below about 50 micromolar. More preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 30 micromolar. Even more preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 20 micromolar. In an even more preferred embodiment, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 10 micromolar. Even more preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 5 micromolar. Even more preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is below about 1 micromolar. Most preferably, the blood plasma concentration of the HMG-CoA reductase inhibitor or its active metabolite at steady-state is about 0.5 micromolar.

Figure 7 shows the steady-state plasma concentrations (nanograms/ml) of lovastatin acid in patients after multiple oral 40 mg doses of Lovastatin XL, a preferred extended release tablet form of lovastatin. Accordingly, based on a conversion factor and the known linear pharmacokinetics of lovastatin it can be expected that oral doses of about 233 mg Lovastatin XL ("Lovastatin XL" refers to a lovastatin controlled release formulation as exemplified herein below) given daily to a patient would result in average blood plasma level of the patient being about 0.5 micromolar.

However, the present inventors have surprisingly discovered that human patients given oral doses of only 10 mg/day, 20 mg/day, 40 mg/day and 60 mg/day of Lovastatin XL resulted in a statistically significant decrease in Aβ peptide levels in the blood plasma of those patients. Accordingly, the inventors have unexpectedly found that the HMG-CoA reductase inhibitor may be administered to a human orally at daily doses of about 10 mg to about 60 mg.

Preferably, the HMG-CoA reductase inhibitor is administered to the mammal orally at a daily dose of about 0.2 mg to 10.0 mg per kg of body weight, given in divided doses. The HMG-CoA reductase inhibitors may be administered in any suitable form. For example, the HMG-CoA reductase inhibitor may be administered in the form of tablets, capsules or oral concentrates suitable for mixing the particular compound with food.

The criteria for the diagnosis of Alzheimer's disease is well known and is set forth in the guidelines of the National Institute of Neurological and Communicative Disorders and Alzheimer's Disease and Related Disorders Association (McKhann et al., Neurology 1984; 34: 939-944); and in the American Psychiatric Association, Diagnostic and Statistical Manual of Mental Disorders (Diagnostic and Statistical Manual IV), all of which are incorporated herein by reference. Generally the objective criteria for the diagnosis of Alzheimer's disease include: gradual memory impairment and gradual onset of at least one of the following aphasia, apraxia, agnosia or disturbance of executive functioning.

Treatment may be continued until there is a reduction in the symptoms of Alzheimer's disease and the dosage may be adjusted in response to the mammal's

individual response. Generally a positive response will not be seen until therapy has been continued for a minimum period of 90 to 365 days.

More preferably, a controlled release formulation (also herein after referred to as a "controlled release composition") of the HMG-CoA reductase inhibitor is utilized in order to provide an enhanced effect that cannot be achieved by conventional immediate release dosing. The use of a controlled release form may be specially useful for providing a constant level of the HMG-CoA reductase inhibitor in order to avoid dosage peaks and valleys in those mammals who have meals at irregular times or those who frequently eat snacks between meals.

Controlled release formulations have been described in U.S. Pat. No. 4,615,698 which have been based on an osmotic dosage form which is designed to collapse and cause the faced surfaces to come into a closed contacting arrangement as the drug is delivered through a passageway in the semi-permeable wall of the dosage form. In addition, U.S. Pat. No. 4,503,030 discloses an osmotic dosage form which has a passageway and a semi-permeable membrane consisting of a particular cellulose polymer and a pH sensitive material which could be an enteric coating material. This patent describes the use of 1:1 mixtures of a pH sensitive material and cellulose polymer which are applied at a level of about 7% by weight based on the total weight of the osmotic core tablet and coating material. The aforementioned patents are incorporated herein by reference.

Preferred HMG-CoA Reductase Inhibitor Formulations

A preferred controlled release formulation is disclosed in U.S. Pat. No. 5,916,595, which is incorporated herein by reference. This type of a controlled release dosage form is preferably prepared by combining the HMG-CoA reductase inhibitor

with a pharmaceutically acceptable, water swellable polymer and an osmotic agent into a compressed tablet core having an optional first coating for sealing and protection and a second coating comprising a pH sensitive agent water insoluble polymer. More preferably, the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, and lovastatin and the active metabolite forms thereof. Even more preferably, the HMG-CoA reductase inhibitor comprises lovastatin or its active metabolite, lovastatin acid. Mevastatin, pravastatin, simvastatin, atorvastatin, and lovastatin are well known compounds that are described in the prior art including the particular patents which have been cited herein. It is also within the scope of the invention to use mixtures of different alkyl esters of hydroxy substituted naphthalenes.

Specifically, the pharmaceutically acceptable, water swellable polymer and the osmotic agent are combined with the HMG-CoA reductase inhibitor which may be micronized, comicronized or unmicronized or amorphous or crystalline and compressed to form the tablet core. The osmotic agent is any suitable non-toxic pharmaceutically acceptable water soluble compound which will dissolve sufficiently in water and increase the osmotic pressure inside the simple sugars and salts such as sodium chloride, potassium chloride, magnesium sulfate, magnesium chloride, sodium sulfate, lithium sulfate, urea, inositol, sucrose, lactose, glucose, sorbitol, fructose, mannitol, dextrose, magnesium succinate, potassium acid phosphate and the like. The preferred osmotic agent for the tablet core is a simple sugar such as anhydrous lactose in the range of about 0-50% by weight, based on the weight of the compressed, uncoated tablet.

The pharmaceutically acceptable, water swellable polymer may be any pharmaceutically acceptable polymer which swells and expands in the presence of water to slowly release the HMG-CoA reductase inhibitor. These polymers include polyethylene oxide, methylcellulose, hydroxypropylcellulose, hydroxypropylmethylcellulose and the like.

In a preferred embodiment, the water swellable polymer will be polyethylene oxide (obtained from Union Carbide Corporation under the trade name Polyox WSR Coagulant or Polyox WSR N 80). These materials form a viscous gel in water or other solvent system at a sufficient concentration to control the release of the HMG-CoA reductase inhibitor. This will generally require a concentration of the pharmaceutically acceptable water swellable polymer of about 0-50% by weight of the compressed, uncoated tablet.

Any suitable binder may be employed. Preferably, the binder is used in a sufficient amount so that when it is combined with a suitable solvent, mixed with the water soluble osmotic agent and agitated, granules will be formed which may be compressed into a tablet core. Prior to compressing the granules, the conventional solid pharmaceutical diluents such as microcrystalline cellulose, lactose, dextrose and the like may be added to the granule based on the weight of the compressed, uncoated tablet. In the present case, the above mentioned osmotic agent, lactose, may function as a binder in the tablet compression step.

In the preparation of the tablets, any suitable solvent may be used to prepare the aforementioned granules. In addition, various other suitable diluents, excipients, lubricants, dyes, pigments, dispersants, emulsifiers, and the like may be used to optimize the HMG-CoA reductase inhibitor formulation.

Additionally, any suitable surfactant may be used. The surfactant may be any ionic or non-ionic water soluble surfactant which is preferably employed in the range of about 0-50% by weight and more preferably employed in the range of about 1-5% by weight. The preferred surfactant for the present formulation is sodium lauryl sulfate but other surfactants such as polysorbate 20, 60, or 80; polyoxl 40 stearate and the like may be used.

Furthermore, a tabletizing formulation may also include any suitable lubricant. Ideally, the lubricant will be in the range of from about 0.5 to about 2.5% by weight of the compressed, uncoated tablet.

After the above described tablet core is formed, it is preferably coated with: 1) an optional protective first coating on the tablet core and/or an optional pH sensitive coating; and 2) an outer coating comprising a pH sensitive agent and a water insoluble polymer.

Specifically, a protective first coating may be used at a level in the range of about 0-10% by weight which may be applied from a coating system such as OPADRY CLEAR™ sold by Colorcon Corporation. In an especially preferred embodiment, the OPADRY CLEAR™ will be about 2.83% by weight and will be combined with an osmotic agent in the range of about 0-10% by weight. While the osmotic agent may be any suitable salt, low molecular weight molecule or water soluble polymer, the preferred osmotic agent is sodium chloride. Preferably, the osmotic agent is added to the coating system when the coating system is being dispersed into purified water. The coating system which contains the osmotic agent may then be sprayed onto the tablets to form a protective coating layer.

An optional inner or over coat over the outer coat may also be applied which comprises a pH sensitive polymer which functions as an enteric polymer in that it does not begin to dissolve until pH conditions in excess of the stomach region are encountered. Generally, the pH sensitive materials do not dissolve and begin to release the active drug until the pH is about 3.0, and preferably above about 5.5. Materials such as Eudragit L (copolymer of poly(methacrylic acid, methylmethacrylate), 1:1 ratio; MW (No. Av. 135,000 - USP Type A) or Eudragit S (copolymer of poly(methacrylic acid, methylmethacrylate, 1:2 ratio MW (No. Av. 135,000 – USP Type B) may be used. Hydroxypropyl methyl cellulose phthalate and the like may be used in the range of about 0-30% by weight and preferably about 2 to about 4% by weight of the combined weight of the compressed, uncoated tablet and the inner coating of the pH sensitive polymer.

Preferably, the outer coating comprises a pH sensitive polymer which functions as an enteric polymer in that it does not begin to dissolve until pH conditions in excess of the pH of the stomach region are encountered and a water insoluble polymer which provide controlled release properties to the coating formulation. The pH sensitive polymer is preferably the same type of material that is described above as the optional inner coating layer. The water insoluble polymer may be a cellulosic polymer such as ethylcellulose, cellulose acrylate, cellulose mono-, di- or triacetate. The pH sensitive polymer and the insoluble cellulosic polymer are used at a weight ratio of about 0.1:1 to about 0.75:1, preferably about 0.25:1 to about 0.5:1 of pH sensitive polymer to water insoluble cellulosic polymer. A combined coating weight of about 0.5-5% by weight and preferably about 1-4% by weight and especially preferred is about 1-3% by weight of the gained weight based on the weight of the coated tablet core. Cellulose

acetate is the preferred water insoluble polymer and the outer coating is preferably applied as a suspension in acetone.

Furthermore, any suitable plasticizer or combination of plasticizers may be added to the inner, outer or over coating to provide elasticity and shape to the coating. While the plasticizer or combination of plasticizers may be any water soluble or water insoluble formulation in the range of about 0-10% by weight and preferably about 0.5-5% by weight of the outer coating composition. Acetyltributyl citrate is the preferred plasticizer but material such as acetyl triethyl citrate, dibutyl phthalate, triacetin, diethyl phthalate, polyethylene glycol, propylene glycol and the like may be utilized.

Any suitable antioxidant such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) may be added to the tablet core as a stabilizer at a level of about 0.001-0.01 % by weight of the tablet core.

Any suitable channeling agent may be mixed with the aforementioned components of the outer coating. A channeling agent may be employed to increase the porosity of the film coating in order to increase the amount of the fluids that penetrate the tablet core and increase the rate of hydration. This allows the release of the HMG-CoA reductase inhibitor after the outer film coat ruptures. Generally, channeling agents may be any salts, surfactants, or short-chain water soluble polymers in a water channel forming effective amount, i.e., about 1-5% by weight, based on the total weight of the core and all coating components. The channeling agents include any pharmaceutically acceptable water soluble salt, surfactant, or short-chain water soluble polymer such as sodium chloride, potassium chloride, sucrose, polysorbate-80, hydroxypropyl cellulose, hydroxyethyl cellulose and the like.

Also, the inner or over coating may be supplied with an anti-sticking agent such as talc to overcome any tablet to tablet stickiness during the coating process. The amount of anti-sticking agent supplied is preferably in an amount which prevents sticking, more preferably in the range of about 0-6% by weight based on the weight of the tablets and the coating materials on a dry weight basis.

The tablets may be made by any suitable method, for example, in a smooth faced tablet die. Thereafter the tablet is preferably provided with the outer coating, which because of surface tension, will result in a thinner coating layer over the corners of the tablet which will provide an area in the outer coating which will form a channel to allow intestinal fluid to reach the core of the tablet.

A preferred control release tablet useful in the practice of the present invention will have the following general formula as set forth in Table 1:

Table 1

Tablet Core:	
Alkyl ester of a substitute naphthalene	3-20 wt%
Water Swellable Polymer	10-40 wt%
Antioxidant	0.001-0.01 wt%
Osmotic Agents	20-80 wt%
Surfactant	0-5 wt%
Lubricant	0-5 wt%
Coatings:	
Seal Coating	0-10 wt%
Osmotic Agents	0-10 wt%
Inner Coating:	
Enteric Polymer	0-30 wt%
Anti-sticking Agent	0-6 wt%
Plasticizer	0-6 wt%
Channeling Agents	0-6 wt%
Outer Coating:	
Blend of Enteric Polymer and Water-	0.5-5 wt%

insoluble Polymer	
Plasticizer(s)	0-1 wt%
Channeling Agents	0.2-5 wt%
Overcoat:	
Enteric Polymer	0-30 wt%
Anti-sticking Agent	0-6 wt%
Plasticizer	0-6 wt%
Channeling Agents	0-6 wt%
TOTAL	100 wt%

A particularly preferred tablet which is useful in the practice of the invention has the ingredients as set forth in Table 2 and may be prepared as set forth below:

Table 2

Lovastatin	12.14 wt%	20.00 mg
Polyox WSR Coagulant, NF (polyethylene oxide Mw No. AV 5,000,000)	4.55 wt%	7.50 mg
Polyox WSR N 80, NF (polyethylene oxide Mw No. AV 200,000)	17.76 wt%	29.25 mg
Lactose (anhydrous)	51.30 wt%	84.50 mg
Sodium lauryl sulfate	3.04 wt%	5.00 mg
Cab-O-Sil (Silicon dioxide Fumed US/NF)	0.46 wt%	0.75 mg
Butylated hydroxy anisole	0.03 wt%	0.05 mg
Myvaplex 600P (glyceryl monostearate)	1.82 wt%	3.00 mg
Seal Coating:		
Opadry Clear (mixture containing hydroxypropyl methyl cellulose and polyethylene glycol)	3.42 wt%	5.63 mg
Sodium chloride	1.14 wt%	1.88 mg
Outer Coating:		
Cellulose acetate	1.43 wt%	2.36 mg
Eudragit S 100 (poly(methylacrylic acid) methylacrylate) 1:2 ratio MW (No. Av. 135,000 – USP Type B)	0.49 wt%	0.80 mg
Triacetin (Glycerol Triacetate)	0.11 wt%	0.19 mg
Polyethylene glycol 400	0.11 wt%	0.19 mg
Sugar, confectioners 6X micronized	0.72 wt%	1.18 mg
Overcoat:		

Hydroxypropylmethylcell. Phthal. 55	0.77 wt%	1.27 mg
Talc	0.30 wt%	0.49 mg
Acetyl tributyl citrate	0.12 wt%	0.20 mg
Sugar, confectioners 6X micronized	0.30 wt%	0.49 mg
	100.0 wt%	146.73 mg

The following describes the preferred process of making the above described dosage form:

Step 1. The tablet core

(a) Granulation

1. Pass Polyox WSR N80, sodium lauryl sulfate and anhydrous lactose through a 30 mesh stainless steel screen.
2. Charge the screened materials and lovastatin (micronized) into a vertical granulator.
3. Prepare a butylated hydroxy anisole solution by dissolving butylated hydroxy anisole in ethanol.
4. Prepare a mixture of ethanol and purified water.
5. Pre-mix the powder mixture from above (step 1(a)2) for 5 minutes.
6. Blend the powder mixture again, add the butylated hydroxyanisole solution and then the ethanol/water mixture.
7. Dry the resulting granules at 45-50 °C until the moisture content is lower than 1.8 wt%.
8. Pass the granules through a 1575 mesh using a Comil.

(b) Tabletting

1. Mix Cab-O-Sil and Polyox WSR N80.

2. Pass the mixture of Cab-O-Sil and Polyox WSR N80 through a 24 mesh stainless steel screen with the Polyox WSR Coagulant.
3. Blend the screened materials with lovastatin granules for 15 minutes.
4. Pass Myvaplex through a 30 mesh stainless steel screen and combine with the other screen materials.
5. Blend for five minutes.
6. Compress the blend into tablets (164.72 mg, round, standard concave, 17/64" dia.) which contain 20 mg of lovastatin.

(c) Seal Coating: Opadry Clear

1. Dissolve sodium chloride in purified water.
2. Disperse Opadry Clear into the sodium chloride solution.
3. Spray lovastatin tablets with the aqueous coating suspension using a coater.

(d) Inner Coating: None

(e) Outer Coating: cellulose acetate

1. Dissolve cellulose acetate and Eudragit S100 in acetone using a homogenizer.
2. Add polyethylene glycol 400, triacetein and sugar to the solution and mix until a homogenous dispersion is obtained.
3. Spray the coating suspension onto the tablets in a coater.

(f) Overcoating: Hydroxypropyl methylcellulose phthalene 55 (HPMCP 55)

1. Dissolve hydroxypropyl methylcellulose phthalene 55 in acetone using a homogenizer.
2. Add acetyl tributyl citrate to the acetone solution and mix it with a homogenizer until a homogenized dispersion is obtained.

3. Add talc and sugar to the solution and mix it with a homogenizer until a homogenized dispersion is obtained.
4. Replace the homogenizer with a magnetic mixer and stir the coating mixture throughout the coating process.
5. Spray the Opadry Clear coated lovastatin tablets with the coating dispersion in a coater.

Other particularly preferred control release tablets useful in the practice of the present invention are those disclosed in U.S. Patent Application Serial No. 09/435,576, which is herein incorporated by reference.

For example, a particularly preferred tablet which is useful in the practice of the present invention has the ingredients as set forth in Table 3 and may be prepared as set forth below:

Table 3		
Lovastatin	11.99 wt%	40.0 mg
Polyox WSR Coagulant, NF (polyethylene oxide Mw No. AV 5,000,000)	4.50 wt%	15.0 mg
Polyox WSR N 80, NF (polyethylene oxide Mw No. AV 200,000)	17.98 wt%	60.0 mg
Lactose (anhydrous)	50.65 wt%	169.0 mg
Sodium lauryl sulfate	3.00 wt%	10.0 mg
Silicon dioxide Fumed USP/NF	0.45 wt%	1.5 mg
Myvaplex 600P (glyceryl monostearate)	1.80 wt%	6.0 mg
Seal Coating:		
Opadry Clear (mixture containing hydroxypropyl methyl cellulose and polyethylene glycol	2.81 wt%	9.4 mg
Sodium chloride	0.93 wt%	3.1 mg
Inner Coating:		
Hydroxypropylmethylcell.phthal.55	2.27 wt%	7.58 mg
Talc	0.78 wt%	2.60 mg
Acetyl tributyl citrate	0.22 wt%	0.75 mg
Sugar, confectioners 6X micronized	0.62 wt%	2.08 mg
Outer Coating:		

Cellulose acetate	1.00 wt%	3.32 mg
Eudragit S 100 (poly(methacrylic acid), methylmethacrylate, 1:2 ratio MW (No. Av. 135,000 – USP Type B)	0.34 wt%	1.13 mg
Triacetin (Glycol Triacetate)	0.08 wt%	0.27 mg
Polyethylene glycol 400	0.08 wt%	0.27 mg
Sugar, confectioners 6X micronized	0.50 wt%	1.66 mg
TOTAL	100.00 wt%	333.66 mg

The following describes a suitable process of making the above described dosage form:

Granulation

1. Pass Polyox WSR N80, sodium lauryl sulfate and anhydrous lactose through a 30 mesh stainless steel screen.
2. Charge the screened materials and lovastatin (micronized) into a vertical granulator.
3. Dissolve buylated hydroxy anisole in ethanol.
4. Mix ethanol, and purified water.
5. Pre-mix the powder mixture for 5 minutes.
6. Blend the powder mixture again, add the butylated hydroxyanisole solution and then the ethanol/water mixture.
7. Dry the granules at 45-50°C until the moisture content is lower than 1.8 wt%.
8. Pass the granules through a 1575 mesh using a Comil.

Tabletting

1. Mix Cab-O-Sil and Polyox WSR N80.

2. Pass the mixture of Cab-O-Sil and Polyvox WSR N80 through a 24 mesh stainless steel screen with the Polyox WSR Coagulant.
3. Blend the screen materials with lovastatin granules for 15 minutes.
4. Pass Myvaplex through a 30 mesh stainless steel screen and combine with the other screen materials.
5. Blend for five minutes.
6. Compress the blend into tablets (300 mg, round standard concave, 11/32") which contain 40 mg of lovastatin.

Seal Coating: Opadry Clear

1. Dissolve sodium chloride in purified water.
2. Disperse Opadry Clear into the sodium chloride solution.
3. Spray lovastatin tablets with the aqueous coating suspension using a coater.

Inner Coating: Hydroxypropyl methylcellulose phthalate 55

1. Dissolve hydroxypropyl methylcellulose phthalate 55 in acetone using a homogenizer.
2. Add acetyl tributyl citrate to the acetone solution and mix it with a homogenizer until a homogenized dispersion is obtained.
3. Add talc and sugar to the solution and mix it with a homogenizer until a homogenized dispersion is obtained.
4. Replace the homogenizer with a magnetic mixer and stir the coating mixture throughout the coating process.
5. Spray the Opadry Clear coated lovastatin tablets with the coating dispersion in a coater.

Outer Coating: cellulose acetate

1. Dissolve cellulose acetate and Eudragit S100 in acetone using a homogenizer.
2. Add polyethylene glycol 400, triactein and sugar to the solution and mix until a homogeneous dispersion is obtained.
3. Spray the coating suspension onto the tablets in a coater.

Another example of a particularly preferred tablet has the ingredients as set forth in Table 4:

Table 4		
Lovastatin	12.11 wt%	40.0 mg
Polyox WSR Coagulant, NF (polyethylene oxide Mw No av 5,000,000)	4.54 wt%	15.0 mg
Polyox WSR N 80, NF (polyethylene oxide Mw No av 200,000)	17.71 wt%	58.5 mg
Lactose (anhydrous)	51.13 wt%	168.9 mg
Sodium lauryl sulfate	3.03 wt%	10.0 mg
Cab-O-Sil (Silicon dioxide Fumed USP/NF)	0.45 wt%	1.5 mg
Butylated hydroxy anisole	0.03 wt%	0.10 mg
Myvaplex 600P (glyceryl monostearate)	1.82 wt%	6.0 mg
Seal Coating:		
Opadry Clear (mixture containing hydroxypropyl methyl cellulose and polyethylene glycol	2.85 wt%	9.4 mg
Sodium Chloride	0.94 wt%	3.1 mg
Inner Coating:		
Hydroxypropylmethylcell.phthal.55	2.29 wt%	7.58 mg
Talc	0.79 wt%	2.6 mg
Acetyl tributyl citrate	0.23 wt%	0.75 mg
Sugar, confectioners 6X micronized	0.08 wt%	0.27 mg
Outer Coating:		
Cellulose acetate	1.00 wt%	3.32 mg
Eudragit S 100 (polymethacrylicacid, methylmethaceylate, 1:2 ratio MW (No. AV. 135,000 – USP Type B)	0.34 wt%	1.13 mg
Triacetin	0.08 wt%	0.27 mg
Polyethylene glycol 400	0.08 wt%	0.27 mg

Sugar, confectioners 6X micronized	0.50 wt%	1.66 mg
TOTAL	100.00 wt%	330.35 mg

The preferred tablet having the ingredients as set forth in Table 4 may be prepared as described above for the preparation of the preferred tablet having the ingredients as set forth in Table 3.

Another example of a particularly preferred tablet has the ingredients as set forth in Table 5 and may be prepared as set forth below:

Table 5

Lovastatin	12.14 wt%	20.0 mg
Polyox WSR Coagulant, NF (polyethylene oxide Mw No. AV 5,000,000)	4.55 wt%	7.5 mg
Polyox WSR N 80, NF (polyethylene oxide Mw No. AV 200,000)	17.76 wt%	29.25 mg
Lactose (anhydrous)	51.30 wt%	84.5 mg
Sodium lauryl sulfate	3.04 wt%	5.0 mg
Cab-O-Sil (Silicon dioxide Fumed USP/NF)	0.46 wt%	0.75 mg
Butylated hydroxy anisole	0.03 wt%	0.05 mg
Myvaplex 600P (glyceryl monostearate)	1.82 wt%	3.0 mg
Seal Coating:		
Opadry Clear (mixture containing hydroxypropyl methyl cellulose and polyethylene glycol)	3.42 wt%	5.63 mg
Sodium chloride	1.14 wt%	1.88 mg
Outer Coating:		
Cellulose acetate	1.43 wt%	2.36 mg
Eudragit S 100 (polymethylacrylic acid, methylacrylate, 1:2 ratio MW (No. Av. 135,000 – USP Type B)	0.49 wt%	0.80 mg
Triacetin	0.11 wt%	0.19 mg
Polyethylene glycol 400	0.11 wt%	0.19 mg
Sugar, confectioners 6X micronized	0.72 wt%	1.18 mg
Overcoat:		
Hydroxypropylmethylcell. Phthal. 55	0.77 wt%	1.27 mg
Talc	0.30 wt%	0.49 mg

Triacetin	0.12 wt%	0.20 mg
Sugar, confectioners 6X micronized	0.30 wt%	0.49 mg
TOTAL	100.0 wt%	146.73 mg

The following describes the process of making the above described dosage form:

Granulation

1. Pass Polyox WSR N80, sodium lauryl sulfate and anhydrous lactose through a 30 mesh stainless steel screen.
2. Charge the screened materials and lovastatin (micronized) into a vertical granulator.
3. Dissolve butylated hydroxy anisole in ethanol.
4. Mix ethanol and purified water.
5. Pre-mix the powder mixture for 5 minutes.
6. Blend the powder mixture again, add the butylated hydroxyanisole solution and then the ethanol/water mixture.
7. Dry the granules at 45-50°C until the moisture content is lower than 1.8 wt%.
8. Pass the granules through a 1575 mesh using a Comil.

Tabletting

1. Mix Cab-O-Sil and Polyox WSR N8O.
2. Pass the mixture of Cab-O-Sil and Polyox WSR N80 through a 24 mesh stainless steel screen with the Polyox WSR Coagulant.
3. Blend the screen materials with lovastatin granules for 15 minutes.

4. Pass Myvaplex through a 30 mesh stainless steel screen and combine with the other screen materials.
5. Blend for five minutes.
6. Compress the blend into tablets (164.72 mg, round, standard concave, 17/6411 dia.) which contain 20 mg of lovastatin.

Seal Coating: Opadry Clear

1. Dissolve sodium chloride in purified water.
2. Disperse Opadry Clear into the sodium chloride solution.
3. Spray lovastatin tablets with the aqueous coating suspension using a coater.

Inner Coating: None

Outer Coating: Cellulose acetate

1. Dissolve cellulose acetate and Eudragit S100 in acetone using a homogenizer.
2. Add polyethylene glycol 400, triactein and sugar to the solution and mix until a homogeneous dispersion is obtained.
3. Spray the coating suspension onto the tablets in a coater.

Overcoating: Hydroxypropyl methylcellulose phthalate 55

1. Dissolve hydroxypropyl methylcellulose phthalate 55 in acetone using a homogenizer.
2. Add acetyl tributyl citrate to the acetone solution and mix it with a homogenizer until a homogenized dispersion is obtained.
3. Add talc and sugar to the solution and mix it with a homogenizer until a homogenized dispersion is obtained.

4. Replace the homogenizer with a magnetic mixer and stir the coating mixture throughout the coating process.
5. Spray the Opadry Clear coated lovastatin tablets with the coating dispersion in a coater.

Another example of a particularly preferred tablet has the ingredients as set forth in Table 6 and may be prepared by the same general procedure as described above for the preparation of the tablet having the ingredients as set forth in Table 5, except that no inner coating is applied and an outer enteric coating is applied as an overcoat over the outer layer.

Table 6

Lovastatin	12.20 wt.%	20.0 mg.
Polyox WSR Coagulant, NF (Polyethylene oxide Mw No av 5,000,000)	4.57 wt.%	7.5 mg.
Polyox WSR N 80, NF (polyethylene oxide Mw No av 200,000)	17.84 wt/%	29.25 mg.
Lactose (anhydrous)	51.53 wt.%	84.5 mg.
Sodium lauryl sulfate	3.05 wt.%	5.0 mg.
Silicon dioxide fumed USP/NF	0.46 wt.%	0.75 mg.
Butylated hydroxy anisole	0.03 wt.%	0.05 mg.
Myvaplex 600P (glyceryl monostearate)	1.83 wt.%	3.0 mg.
Seal Coating:		
Opadry Clear (mixture containing hydroxypropyl methyl cellulose and polyethylene glycol)	3.43 wt.%	5.63 mg.
Sodium chloride	1.15 wt.%	1.88 mg.
Inner Coating: None		
Outer Coating:		
Cellulose acetate	1.96 wt.%	3.21 mg.
Eudragit S 100	0.66 wt.%	1.09 mg.
Acetyl tributyl citrate	0.32 wt.%	0.52 mg.
Sugar, confectioners 6X micronized	0.98 wt.%	1.61 mg.
TOTAL	100.00 wt.%	163.99 mg.

Other examples of particularly preferred 40 mg. tablets have the ingredients as set forth in Table 7 and may be prepared by the same method described above for preparing the tablet having the ingredients as set forth in Table 3.

Table 7			
Summary of Lovastatin Formulations			
Ingredient	Weight Percent		
	Tablet A	Tablet B	Tablet C
Lovastatin (strength, mg)	40 wt.%	40 mg.	40 mg.
Tablet Core			
1. Lovastatin	12.11 wt.%	12.28 wt.%	12.28 wt.%
2. Lactose (Anhydrous)	51.13 wt.%	51.8 wt.%	51.8 wt.%
3. Polyox® WSR Coagulant	4.54 wt.%	4.6 wt.%	4.6 wt.%
4. Polyox® WSR N80	17.71 wt.%	17.94 wt.%	17.94 wt/%
5. Sodium Lauryl Sulfate	3.03 wt.%	3.06 wt.%	3.06 wt.%
6. Glyceryl Monostearate	1.82 wt.%	1.84 wt.%	1.84 wt.%
7. Silicon Dioxide	0.45 wt.%	0.46 wt.%	0.46 wt.%
8. Butylated Hydroxyanisole	0.03 wt.%	0.02 wt.%	0.02 wt.%
Seal Coat			
1. Opadry Clear	2.85 wt.%	2.88 wt.%	2.88 wt.%
2. Sodium Chloride Powder	0.94 wt.%	0.96 wt.%	0.96 wt.%
Inner Coat			
1. HPMCP 55	2.29 wt.%	1.61 wt.%	1.61 wt.%
2. Talc, USP	0.79 wt.%	0.55 wt.%	0.55 wt.%
3. Acetyltributyl Citrate	0.23 wt.%	0.16 wt.%	0.16 wt.%
4. Sugar, Micronized	0.64 wt.%	0.44 wt.%	0.44 wt.%
Outer Coat			
1. Cellulose Acetate	1 wt.%	0.7 wt.%	0.7 wt.%
2. Eudragit S100	0.34 wt.%	0.24 wt.%	0.24 wt.%
3. Triacetin	0.08 wt.%	0.06 wt.%	0.06 wt.%
4. Polyethylene Glycol 400	0.08 wt.%	0.6 wt.%	0.6 wt.%
5. Acetyltributyl Citrate	-	-	-
6. Sugar, Micronized	0.5 wt.%	0.35 wt.%	0.35 wt.%
Overcoat			
1. HPMCP 55	-	-	-
2. Talc, USP	-	-	-
3. Triacetin	-	-	-
4. Sugar, Micronized	-	-	-
5. Opadry Yellow	-	-	-
6. Opadry Pink	-	-	-
TOTAL TABLET WEIGHT, %	100 wt.%	100 wt.%	100 wt.%

Examples of other preferred tablets having the ingredients as set forth in Table 8 may be prepared by the same method described above for preparing the tablet having the ingredients as set forth in Table 3.

Table 8

Summary of Lovastatin Formulations

Ingredient	Weight Percent	
	Tablet D	Tablet E
Lovastatin (strength, mg)	20 mg.	10 mg.
Tablet Core		
1. Lovastatin	11.69 wt.%	5.84 wt.%
2. Lactose (Anhydrous)	49.32 wt.%	55.18 wt.%
3. Polyox® WSR Coagulant	4.38 wt.%	4.38 wt.%
4. Polyox® WSR N80	18.08 wt.%	17.09 wt.%
5. Sodium Lauryl Sulfate	2.92 wt.%	2.92 wt.%
6. Blyceryl Monostearate	1.75 wt.%	1.75 wt.%
7. Silicon Dioxide	0.44 wt.%	0.44 wt.%
8. Butylated Hydroxyanisole	0.02 wt.%	0.01 wt.%
Seal Coat		
1. Opadry Clear	2.74 wt.%	2.74 wt.%
2. Sodium Chloride Powder	0.91 wt.%	0.91 wt.%
Inner Coat		
1. HPMCP 55	2.21 wt.%	2.21 wt.%
2. Talc, USP	0.76 wt.%	0.76 wt.%
3. Acetyltributyl Citrate	0.22 wt.%	0.22 wt.%
4. Sugar, Micronized	0.61 wt.%	0.61 wt.%
Outer Coat		
1. Cellulose Acetate	0.97 wt.%	0.97 wt.%
2. Eudragit S100	0.33 wt.%	0.33 wt.%
3. Triacetin	0.08 wt.%	0.08 wt.%
4. Polyethylene Glycol 400	0.08 wt.%	0.08 wt.%
5. Acetyltributyl Citrate	-	-
6. Sugar, Micronized	0.49 wt.%	0.49 wt.%
Overcoat		
1. HPMCP 55	-	-
2. Talc, USP	-	-
3. Triacetin	-	-
4. Sugar, Micronized	-	-
5. Opadry Yellow	3 wt.%	3 wt.%
6. Opadry Pink	-	-
TOTAL TABLET WEIGHT, %	100 wt.%	100 wt.%

As illustrated in the following examples, cholesterol depletion may lead to a decrease in the release and formation of Aβ peptides in the cells. Additionally, the applicants have discovered that the decreased release of Aβ peptides is not due to the accumulation of the Aβ peptide in the cells, but rather due to the decreased formation of Aβ peptides. Further, the formation APP_s, is also reduced by cholesterol treatment with a HMG-CoA reductase inhibitor, but to a much lesser degree. Further, decreased maturation (glycosylation and sulfation) of APP_i has been excluded as a cause for the effects cholesterol depletion treatment with a HMG-CoA reductase inhibitor on APP_m processing and Aβ peptide formation. Thus, applicants have discovered that reducing cellular cholesterol by the use of an HMG-CoA reductase inhibitor regulates APP_m processing and Aβ formation.

In the following examples, EasyTag™ EXPRESS™ Methionine Protein Labeling Mix, [^{35}S] (spec. activity >1,000 Ci/mMol) was obtained from NEN Life Sciences, Boston, MA; Fetal Calf Lipid Depleted Serum (FCLPDS) was obtained from Intracel, Rockville, MD; Dulbecco's modified Eagles Medium (DMEM) was obtained form BioWittaker, Walkersville, MD; Dulbecco's phosphate buffered saline (PBS) and Fetal Bovine Serum (FBS) were obtained from Life Technologies, Rockville, MD; Antibody 6E10 was obtained from Senetek, Napa, CA; Agarose bound antisera anti-mouse IgG was obtained from American Qualex Antibodies, San Clemente, CA; Protein A sepharose was obtained from Pharmacia Biotech, Piscataway, NJ; Tissue culture plates were obtained from Falcon, Lincoln Park, NJ with the exception of the 10 mm culture dishes with glass coverslips which were obtained from MatTek Corporation, Ashland, NM; and all other chemicals were obtained from Sigma, St. Louis, MO.

The three cell lines utilized were: Chinese Hamster Ovary (CHO) cells expressing the 751 amino acid form of APP; Mabin-Darby Canine Kidney (MDCK) cells which overexpress the 695 amino acid form of APP; Human neuroglioma (H4) cells overexpressing the 695 form of human APP. All cells were prepared by the stable introduction of a cDNA coding for human APP. All cell lines were maintained in DMEM containing 10% FBS and antibiotics.

EXAMPLES

The following examples are intended to illustrate but not to limit the invention.

Example 1

Effects of Cholesterol Depletion

In order to characterize the effects of cholesterol depletion, cell cultures of each cell line were cultured on six-well plates for 4 days in DMEM containing 10% FCLPDS, which lipid depleted medium reduced the external source of cholesterol, in the presence of lovastatin acid (LA) or absence of LA.

In order to confirm that this treatment was sufficient to reduce cellular cholesterol, filipin, a fluorescent dye that binds to cholesterol, was utilized to provide a visual and quantitative measure of the level of cholesterol in the membrane. Generally, the cells were plated onto 10 mm culture dishes in media containing DMEM with 10% FCLPDS and antibiotics. Following incubation, cells were washed once with PBS and fixed with 3% paraformaldehyde in PBS for 1 hour, followed by washing 3 times in PBS for 5 minutes and quenching with 1.5 mg/ml glycine in PBS for 10 minutes. The cells were subsequently stained with 0.5 mg/ml filipin in PBS for 2 hours and washed 3 times for 5 minutes in PBS. After the final wash, the cells were visualized under a fluorescent microscope.

For measurement of APP processing and Aβ peptide formation, medium was removed and the cells were washed once with PBS and then incubated for 2 hours in DMEM containing 1 mCi/ml [^{35}S] Methionine. After this "pulse" period, the cells were either (1) lysed to measure the total labeled APP_i and APP_m at time zero, or (2) the cells were incubated for 2 hours in fresh, unlabeled complete medium ("chase") and then lysed. Then the cell supernatants and lysates were treated with the appropriate antibody to calculate the amounts of APP_i, APP_m, APP_s, and Aβ peptides.

To measure cell-associated, full-length APP_i and APP_m, or to measure carboxyl-terminal fragments of APP, cell lysates were incubated with antibody 369 which recognizes the carboxyl-terminus of APP. *See* Buxbaum, J. D., et al. (1990) *Proc Natl Acad Sci USA* 87:6003-6, which is incorporated herein by reference.

To measure Aβ peptides, or to measure APP_s, which is the secreted carboxyl-terminal truncated form, cell supernatants were incubated with antibody 6E10, which recognizes the first 15 amino acids of the Aβ peptide that correspond to the COOH-terminal amino acids of APP_s. *See* Buxbaum, J. D., et al. (1994) *Proc Natl Acad Sci USA.* 91:4489-93, which is incorporated herein by reference.

The incubations with antibody 6E10 or antibody 369 were performed at 4°C for 75 minutes followed by a 45 minute incubation at 4°C with either agarose-linked anti-mouse IgG for antibody 6E10 or protein A sepharose for antibody 369. The beads were then washed three times for 10 minutes and then run on either a 10-20% Tris-Tricine Gel for APP_s and Aβ peptides or an 8% polyacrylamide gel for cell-associated APP. The gels were dried and exposed to a Phosphor Imager® screen (STORM 860, Molecular Dynamics) and exposed for a minimum of two days. The protein bands

were visualized on a STORM 860 Phosphor Imager® (Molecular Dynamics) and quantitated using ImageQuant® (Molecular Dynamics).

To measure extracellular APP_s and fragments thereof, the cell culture supernatant were utilized. To measure the intracellular APP_i and APP_m and fragments thereof, the cell lysates were utilized.

To determine whether a decrease in cholesterol is associated with a change in the amount of extracellular Aβ peptides, the H4, MDCK, and CHO cells, expressing human APP_m were incubated for 4 days in the presence or absence of 0.5 μM LA. The cells were then incubated in serum-free media containing 1 mCi/ml [^{35}S] Methionine for 2 hours followed by incubation in complete fresh serum-free medium containing unlabeled methionine for an additional 2 hours. The [^{35}S]-labeled Aβ peptides were immunoprecipitated from the cell culture supernatant, resolved by SDS-PAGE, and visualized by autoradiography. *See* Figures 2a, 3a, and 4a. Relative levels of extracellular Aβ peptides were determined under each condition by quantitative PhosphorImager autoradiography. *See* Figures 2b, 3b, and 4b.

By subsequently focusing only on the [^{35}S]-labeled protein, the amounts of [^{35}S]-labeled Aβ peptides were normalized to the levels of total [^{35}S]-labeled APP_i by dividing total [^{35}S]-labeled Aβ by total [^{35}S]-labeled APP_i to exclude any changes in Aβ peptide levels due to decreased synthesis of Aβ peptides. At the end of the 2 hour chase the analysis was restricted to the proteolysis of APP_m and secretion of Aβ peptides. *See* Buxbaum, J. D., et al. (1990) *Proc Natl Acad Sci USA*. 87:6003-6, which is herein incorporated by reference.

As shown in Figures 2a, 2b, 3a, 3b, 4a, and 4b, the amounts of extracellular Aβ peptides in the presence of 0.5 μM LA decreased by 40-60% as compared to the untreated cells. Treatment of cells with 0.5 μM LA had a weaker effect on decreasing extracellular Aβ peptide levels (<20% reduction).

To determine whether the decreased levels of extracellular Aβ peptides observed were due to decreased formation of Aβ peptides, rather than decreased secretion of Aβ peptides from the cells, the levels of [^{35}S]-labeled intracellular Aβ peptides within the cell were measured in the cell lysates. No detectable levels of intracellular Aβ peptides were observed in cells incubated in the presence or absence of 0.5 μM LA. Therefore, the decrease in extracellular Aβ peptides was not due to decreased secretion of Aβ peptides, but instead confirms that it was due to the decreased formation of Aβ peptides from the cleavage of APP_m.

To determine whether cholesterol depletion affects other aspects of APP processing, H4 cells were incubated for four days in the presence or absence of 0.5 μM LA, and subjected to metabolic labeling. The levels of [^{35}S]-labeled APP_i and APP_m were determined by immunoprecipitation from cell lysates with an antibody against the COOH-terminal of APP, followed by quantitative autoradiography. Similarly, the levels of [^{35}S]-labeled extracellular and intracellular Aβ peptides were determined by immunoprecipitation of either cell culture supernatants (extracellular Aβ peptides) or cell lysates (intracellular Aβ peptides). The amounts of each were normalized to the levels of [^{35}S]-labeled APP_i found in cells at the beginning of the chase. Normalization was done by dividing the relevant value by the levels of [^{35}S]-labeled APP_i found in cells at the beginning of the chase.

As illustrated in Figure 5, a modest decrease in [^{35}S]-labeled APP_s formation in cells incubated in the presence of LA, as compared to control cells was observed. Figure 1 is a schematic illustrating APP processing. Because APP_m is likely to be the precursor for both APP_s and Aβ peptides, decreased formation of both APP_s and Aβ peptides might suggest a decrease in the levels of APP_m. To examine this, the levels of [^{35}S]-labeled APP_m was measured in cells incubated in the absence or presence of 0.5 μM LA. As shown in Figure 6, the effects on maturation were not sufficient to account for the decrease in Aβ peptide levels. Therefore, the effects of LA on Aβ peptide levels could not be accounted for by decreased maturation of APP_m and instead reflect effects of LA on the post-Golgi processing or trafficking of APP_m or both.

Example 2

Effective Concentration Range of Lovastatin Acid

To determine the effective range of concentrations of LA, each cell type was grown in the absence or presence of various concentrations of LA by the methods described in Example 1. As shown in the bar graphs in Figures 2a, 2b, 3a, 3b, 4a, and 4b, the amount of extracellular Aβ peptides decreased with increasing LA concentrations and a concentration of 0.05 μM LA or higher was sufficient to significantly ($p<0.001$) decrease the amount of extracellular Aβ peptides under these experimental conditions.

Example 3

Candidate Substance Screening

CHO cells were determined to be suitable for candidate screening because treatment with LA does not affect the maturation of APP_i to APP_m in the CHO cells. Specifically, it was determined that treatment of CHO cells with 0.5 μM LA reduced the amount of extracellular APP_s by about 30% of the amount calculated for the control and reduced the amount of extracellular Aβ peptides by about 70% of the amount calculated for the control when no LA was present. The amount of extracellular Aβ peptides and the amount of extracellular APP_s were normalized to the amount of total APP found in the cell at the end of cell labeling as described above. This normalization provides an effective means of accounting for any differences between cultures and any differences due to altered APP_i synthesis or maturation in cells treated with the candidate compounds. However, the level of total APP_m was comparable between the control and the treated cells (3.2×10^6 arbitrary units and 3.4×10^6 arbitrary units, respectively). This suggests that the maturation of APP_i to APP_m was not affected in the CHO cells under experimental conditions.

Thus, CHO cells and other cells which manufacture Aβ peptides may be used as a suitable screening tool for a candidate substance which affects the synthesis, maturation or post-translational processing of APP. The cells are cultured on six-well plates for 4 days in DMEM containing 10% FCLPDS, which lipid depleted medium reduced the external source of cholesterol, in the presence of the candidate substance or absence of the candidate substance.

Specifically, the CHO cells are pulsed with [^{35}S] Methionine in the absence and presence of the candidate substance. After the pulse period, the cells are either (1) chased for two hours, or (2) lysed to determine the total intracellular APP at time zero.

Then the lysates may be labeled with the appropriate antibody to calculate the amounts of APP_i, APP_m, APP_s, and Aβ peptides.

To measure cell-associated, full-length APP_i and APP_m, or to measure carboxyl-terminal fragments of APP, cell lysates are incubated with antibody 369 which recognizes the carboxyl-terminus of APP. *See* Buxbaum, J. D., et al. (1990) *Proc Natl Acad Sci USA* 87:6003-6, which is incorporated herein by reference.

To measure Aβ peptides, or to measure APP_s, which is the secreted carboxyl-terminal truncated form, cell supernatants are incubated with antibody 6E10, which recognizes the first 15 amino acids of the Aβ peptide that correspond to the COOH-terminal amino acids of APP_s. *See* Buxbaum, J. D., et al. (1994) *Proc Natl Acad Sci USA.* 91:4489-93, which is incorporated herein by reference.

The incubations with antibody 6E10 or antibody 369 are performed at 4°C for 75 minutes followed by a 45 minute incubation at 4°C with either agarose-linked anti-mouse IgG for antibody 6E10 or protein A sepharose for antibody 369. The beads are then washed three times for 10 minutes and then run on either a 10-20% Tris-Ticine Gel for APP_s and Aβ peptides or an 8% polyacrylamide get for cell-associated APP. The gels are dried and exposed to a Phosphor Imager® screen and exposed for a minimum of two days. The protein bands are visualized on a STORM 860 Phosphor Imager® (Molecular Dynamics) and quantitated using ImageQuant (Molecular Dynamics).

To measure extracellular APP_s and fragments thereof, the cell culture supernatant are utilized. To measure the intracellular APP_i and APP_m and fragments thereof, the cell lysates are utilized.

The amount of extracellular Aβ peptides and the amount of extracellular APP_s are normalized to the amount of total APP_i found in the cell at the end of cell labeling as described above. This normalization provides an effective means of accounting for any differences between cultures and any differences due to altered APP_i synthesis or maturation in cells treated with the candidate compounds.

Example 4

Human Trials

A study was conducted to assess the effects of Lovastatin XL on blood lipid levels in patients with hyperlipidemia. Patients were treated with placebo, 10, 20, 40 or 60 mg per day of lovastatin administered as Lovastatin XL. Blood samples were obtained from selected patients prior to dosing and at 1 month after dosing. Because this clinical trial was carried out as part of an New Drug Application, at the time of filing U.S. Provisional Patent Application No. 60/223,987 ("the '987 application"), the inventors were not permitted to determine what dose (placebo, 10, 20, 40 or 60 mg) each of the selected patients where given per day. These blood samples were assayed for Aβ peptide concentrations (pg/ml). The results are listed in Table 9 below:

Table 9 Baseline and 1-Month Beta Amyloid Values

Patient	Baseline Aβ peptide conc. (pg/ml)	Change in Aβ peptide conc. after 1 Month (pg/ml)
1	145.2	-57.8
2	211.1	-30.5
3	151.1	-16.9
4	175.5	60.6
5	388.1	44
6	499.7	-172.7
7	164	-64.1
8	220	-67.5
9	215.5	-80.3
10	370.1	-18.5
11	403.9	-76.2
12	48.9	-32.9
13	15.6	38.4
14	64.6	-13.4
15	34.3	-18
16	12	-1.1
17	45.4	10.9
18	12	4.6
19	37.3	-5.6
20	30.6	0.2
21	35.2	10
22	138.5	-112.5
23	60.6	-38.5
24	50	22
25	73	-22
26	133	-17
27	23	70
28	82	-21
29	4	4
30	56	5
31	59	17
32	2	4
33	3	1
34	181	-42
35	0	5
36	175.4	-37
Mean	120.016667	-18.0222222
SD	126.784261	46.9449472

As can be seen from Table 9, the mean Aβ peptide concentration prior to treatment was 120 pg/ml, which decreased by about 18 pg/ml after one month of treatment with Lovastatin XL. The change from pre-treatment was statistically

significant (p= 0.0273) as shown in Table 10. In the instant case, one of ordinary skill in the art will understand that the above referred to p value represents the probability that the reported change in Aβ peptide concentration could occur by chance. One of ordinary skill in the art will also understand that a p value of less that 0.05 signifies that the reported change is statistically meaningful.

Table 10 Statistical Analysis of Change From Baseline

Results:

Two tailed T-test results:

Variable	MU0	Estimate	Std. Err.	DF	Tstat
var1	0	-18.022223	7.8241577	35	-2.3034072

Variable	Pval
var1	0.0273

Following the filing of the '987 application, the inventors have performed a more detailed analysis of the data obtained from the human trials. Table 11 sets forth a dose-response analysis of the data and shows the mean percentage change in the Aβ peptide concentration in the blood of the patients treatment with placebo, 10, 20, 40 and 60 mg/day Lovastatin XL. Blood samples were taken from the patients after four weeks of treatment (Study Visit No. 5), after eleven weeks of treatment (Study Visit No. 7) and twelve weeks of treatment with Lovastatin XL (Study Visit No. 8).

Table 11 Effect of Various Doses of Lovastatin XL on Aβ Peptide Concentration

DOSE (mg/day)		Study Visit No. 5	Study Visit No. 7	Average of Study Visit Nos. 7 & 8	ENDPOINT
0	N	11	6	8	12
	MEAN	-1.78	-16.27	-21.11	-11.87
	STD	33.69	29.03	23.16	27.03
10	N	10	5	6	10
	MEAN	8.09	43.29	10.21	-0.21
	STD	64.92	72.69	57.83	49.66
20	N	10	5	8	11
	MEAN	0.59	-18.21	-27.81	-21.71
	STD	35.56	43.00	49.91	45.04
40	N	6	3	4	10
	MEAN	-6.51	-6.15	-26.35	-11.92
	STD	22.75	32.05	48.13	46.12
60	N	8	3	7	10
	MEAN	-5.08	-16.27	-35.47	-39.42
	STD	39.78	25.78	27.83	30.09

N—No. of patients
MEAN—mean % change in the Aβ peptide concentration in the blood of patients
STD—Standard deviation

The graph shown in Figure 8 depicts the results set forth in Table 11 above. In particular, the graph shows the change in mean Aβ peptide concentration in the blood of patients after one month of treatment with Lovastatin XL as a function of the dose administered. Figure 8 also includes a "trendline", i.e., the best straight-line approximation, of the data presented in the graph. As can be seen from the "trendline", its direction and slope clearly suggests that the dose of Lovastin XL administered does have an effect on the mean Aβ peptide concentration. The inventors also found that the dose-response analysis for the endpoint values was statistically significant (p=0.0442).

Further human trials have been conducted. Patients meeting the current criteria for treatment with lipid lowering agents were treated with single-blind placebo for 4

weeks. Those patients were then randomly assigned to receive daily doses of 10, 20, 40 or 60 mg/day of a controlled release lovastatin (Lovastatin XL) or matching placebo under double-blind conditions. Serum samples from those patients were obtained prior to and after 3 months of dosing. The serum samples were assayed for Aβ peptide using the assay set forth in Example 5 below. The assay results, expressed as percent change from pre-treatment of serum Aβ peptide concentration levels, are shown in Figure 9.

As can be seen from Figure 9, the placebo treated patients showed a mean increase of serum Aβ peptide concentration levels from baseline, however, this difference was not found to be statistically significant. The mean percent changes in the serum Aβ peptide concentration levels for the patients who received treatment with the controlled release formulation of lovastatin all decreased. These reported percentage changes for the groups of patients treated with 20, 40 and 60 mg/day were determined to be statistically significant $p<0.01$ (t-test). Further, the percentage changes for the groups of patients treated with 40 and 60 mg/day were determined to be statistically significantly different from those of the placebo group, $p<0.05$ (t-test).

Example 5

Aß End-Specific Protocol

The serum samples referred to above in Example 4 were assayed for Aβ peptide using appropriate assays. The assay used for the human trials, the results of which are set forth in Figure 9, was carried out as follows:

Ninety-six well plates (Falcon Probind) were coated with 150 µl of the 4G8 monoclonal antibody (Senetek Crude IgG Ascites Fluid) in carbonate-bicarbonate buffered solution (Sigma) and then incubated at 37°C for 12-16 hours. The plates were

then washed three times with 150 µl/well ECW buffer (PBS, 0.1% BSA, 0.05% Tween-20, 0.2% CHAPS, 5mM ethylenediaminetetraacetic acid, 2mM betaine, 0.05% NaN_3) before adding 150 µl/well ECW buffer containing 1% casein and incubated at 37°C for an additional 4 hours. The 4G8 antibody recognizes Aß and thus selects this peptide from the pool of others in the plasma.

The coated plates were washed twice with 150 µl/well ECW and then 50 µl/well ECW was added to ensure that the wells did not dry out during sample loading.

The standard curves of synthetic Aß 1-40 peptide were prepared by diluting the 100 ng/µl into working solutions using ECW. For the assays the following concentrations were used: 0, 10, 50, 100, 250 and 500 pg/ml synthetic peptide. The standards were loaded in duplicate onto the wells.

Each plasma sample was thawed and then sonicated for 20 seconds prior to loading onto wells in quadruplicate. Each plate contained two internal reference samples and all patient visits were loaded on the same plate.

The loaded plates were incubated for 5 minutes at room temperature, and then for 2 days at 4°C ("capture phase"). The plates were then washed twice with 150 µl/well ECW,150 µl of biotinylated 6E10 monoclonal antibody (Senetek mAbs Biotin 6E10) diluted 1:1000 in ECW was added to each well and the plates incubated at room temperature for 12-15 hours. The 6E10 antibody recognizes Aß which is "captured" by the 4G8 and is biotinylated so that it can be detected by the tertiary antibody.

The plates were washed three times with 150 µl/well ECW before 150 µl of streptavidin alkaline phophatase (Amersham) was added per well and incubated at room temperature for 5 hours. The plates were then washed three times with 150 µl/well ECW before 100 µl/well ddH_20 was added. The water was then aspirated and

100 µl of the Attophos reagent (JBL Scientific Inc) added per well before being allowed to develop at room temperature in the dark. When the highest point in the standard curve began to turn yellow, the plates were read on a microplate reader (PerSeptive Biosystems CytoFluor Series 4000) at an excitation of 450 nm and an emission of 530 nm.

The foregoing description has been presented for purposes of illustration and description. It is not intended to be exhaustive or to limit the invention. The accompanying drawings are included to provide a further understanding of the invention and are incorporated in and constitute a part of this specification, illustrate several embodiments of the invention and together with the description serve to explain the principles of the invention. Obvious modifications or variations are possible in light of the above teachings. All such obvious modification and variations are intended to be within the scope of the present invention.

WHAT IS CLAIMED IS:

1. A method for treating a mammal having an APP processing disorder comprising administering to the mammal a controlled release composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

2. The method of claim 1, wherein the APP processing disorder is Alzheimer's disease or Down's Syndrome.

3. The method of claim 1, wherein the mammal is human.

4. The method of claim 1, wherein the composition further comprises a pharmaceutically acceptable excipient.

5. The method of claim 1, wherein the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, lovastatin, rivastatin, fluvastatin, and pharmaceutically acceptable salts, isomers and active metabolite forms thereof.

6. The method of claim 5, wherein the HMG-CoA reductase inhibitor is lovastatin or lovastatin acid.

7. The method of claim 3, wherein about 10 mg to about 60 mg of the HMG-CoA reductase inhibitor is administered per day.

8. The method of claim 1, wherein about 0.2 mg to about 10 mg of the HMG-CoA reductase inhibitor per Kg of the mammal's body weight is administered per day.

9. The method of claim 1, wherein the composition comprises an amount of the HMG-CoA reductase inhibitor such that the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 50 micromolar.

10. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 40 micromolar.

11. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 30 micromolar.

12. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 20 micromolar.

13. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 10 micromolar.

14. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 5 micromolar.

15. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is below about 1 micromolar.

16. The method of claim 9, wherein the average blood plasma concentration of the HMG-CoA reductase inhibitor or an active metabolite thereof at steady-state is about 0.5 micromolar.

17. A method for treating a mammal having an APP processing disorder comprising lowering the amount of Aβ peptides in the brain, cerebral spinal fluid, or plasma of the mammal by administering to the mammal a controlled release

composition having a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

18. The method of claim 17, wherein lowering the amount of Aβ peptides in the brain comprises affecting APP_m processing.

19. The method of claim 17, wherein the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, lovastatin, rivastatin, fluvastatin, and pharmaceutically acceptable salts, isomers and the active metabolite forms thereof.

20. A method for treating a mammal having an APP processing disorder comprising increasing the clearance of Aβ peptides in the brain, cerebral spinal fluid, or plasma of the mammal by administering to the mammal a controlled release composition having a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

21. The method of claim 20, comprising increasing the clearance of Aβ peptides in the brain of the mammal.

22. The method of claim 20, wherein the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, lovastatin, rivastatin, fluvastatin, and pharmaceutically acceptable salts, isomers and the active metabolite forms thereof.

23. A method for treating a mammal having an APP processing disorder comprising preventing or reducing Aβ peptide aggregation or plaque formation in the brain of the mammal by administering to the mammal a controlled release composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

24. The method of claim 23, wherein the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, lovastatin, rivastatin, fluvastatin, and pharmaceutically acceptable salts, isomers and the active metabolite forms thereof.

25. A method for treating a mammal exhibiting the objective symptoms of Alzheimer's disease by administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

26. The method of claim 25, wherein the HMG-CoA reductase inhibitor decreases the formation of Aβ peptides, increases the clearance of Aβ peptides, regulates the processing of APP, or reduces plaque maturation in the mammal.

27. The method of claim 25, wherein the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin, lovastatin, rivastatin, fluvastatin, and pharmaceutically acceptable salts, isomers and the active metabolite forms thereof.

28. The method of claim 27, wherein the HMG-CoA reductase inhibitor is lovastatin or lovastatin acid.

29. A method for treating a mammal having Down's Syndrome by administering to the mammal a composition comprising a therapeutically effective amount of at least one HMG-CoA reductase inhibitor.

30. The method of claim 29, wherein the HMG-CoA reductase inhibitor decreases the formation of Aβ peptides, increases the clearance of Aβ peptides, regulates the processing of APP, or reduces plaque maturation in the mammal.

31. The method of claim 29, wherein the HMG-CoA reductase inhibitor is selected from the group consisting of mevastatin, pravastatin, simvastatin, atorvastatin,

lovastatin, rivastatin, fluvastatin, and pharmaceutically acceptable salts, isomers and the active metabolite forms thereof.

32. The method of claim 31, wherein the HMG-CoA reductase inhibitor is lovastatin or lovastatin acid.

33. A method for treating a mammal having an APP processing disorder comprising lowering the amount of cellular cholesterol levels in the mammal.

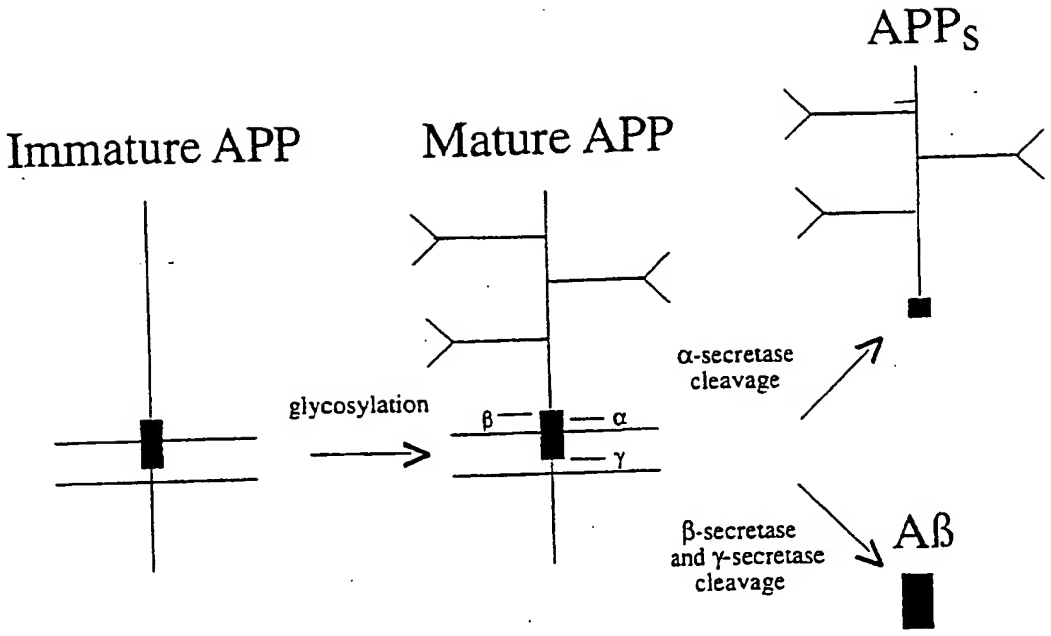

APPs
Immature APP
Mature APP
α-secretase cleavage
glycosylation
β
α
γ
β-secretase and γ-secretase cleavage
Aß

Figure 1

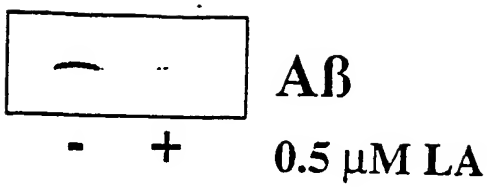


Figure 2a

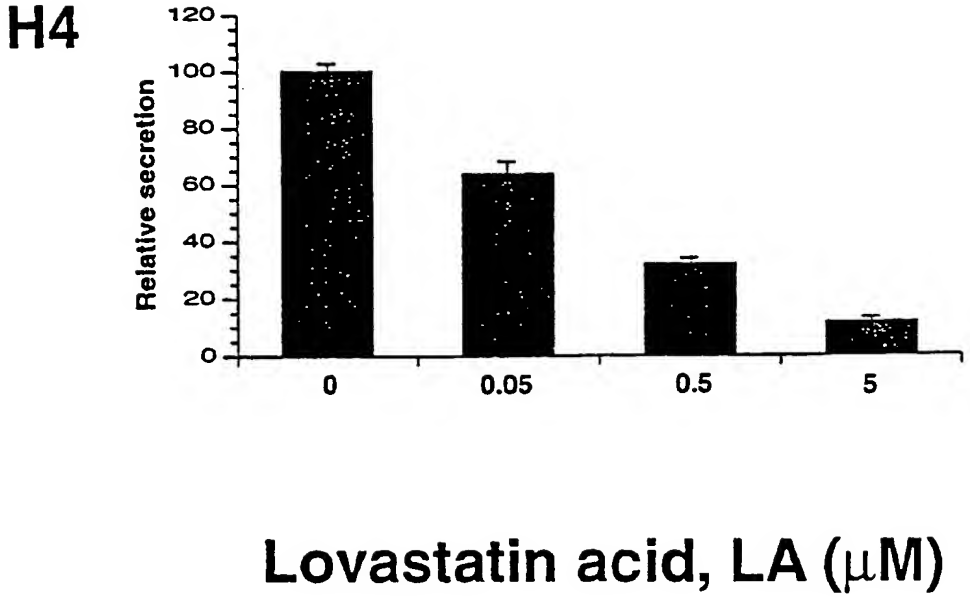


Figure 2b

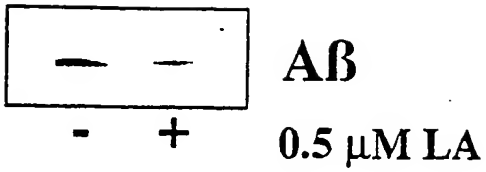


Figure 3a

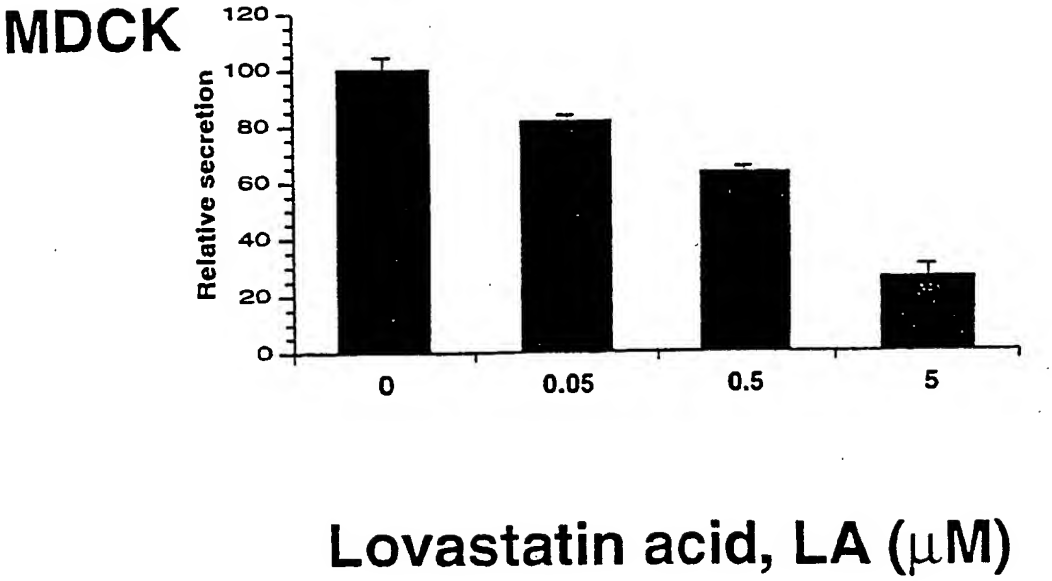


Figure 3b

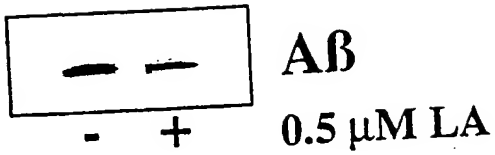


Figure 4a

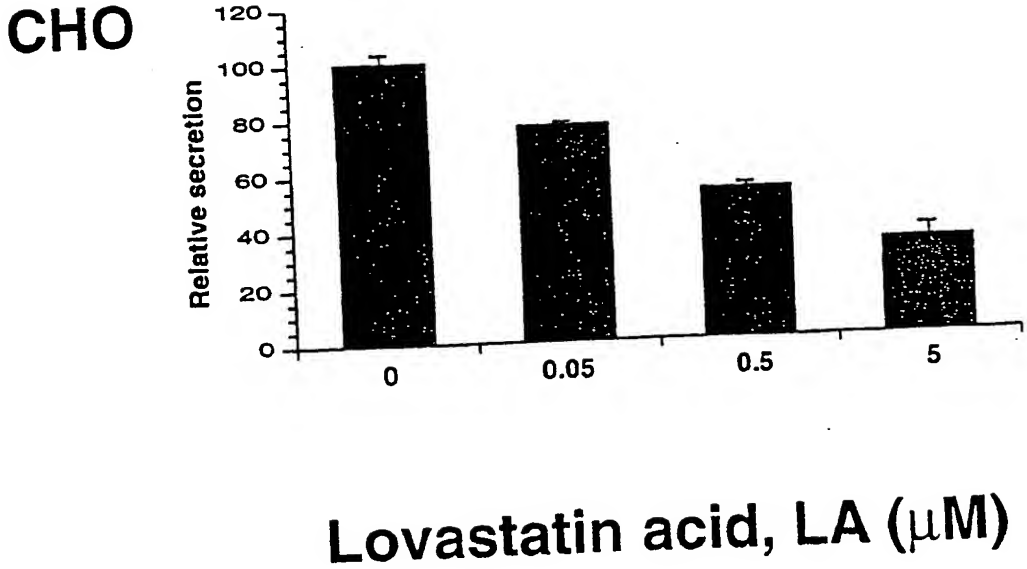
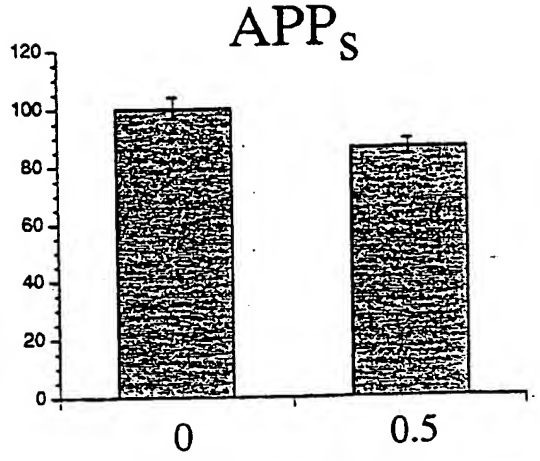


Figure 4b



Lovastatin acid, LA (μM)

Figure 5

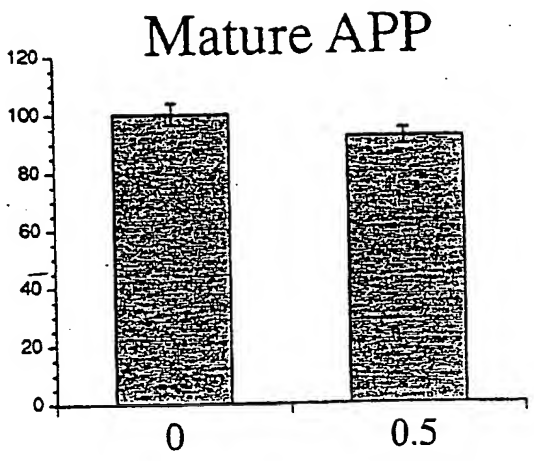


Figure 6

Figure 9

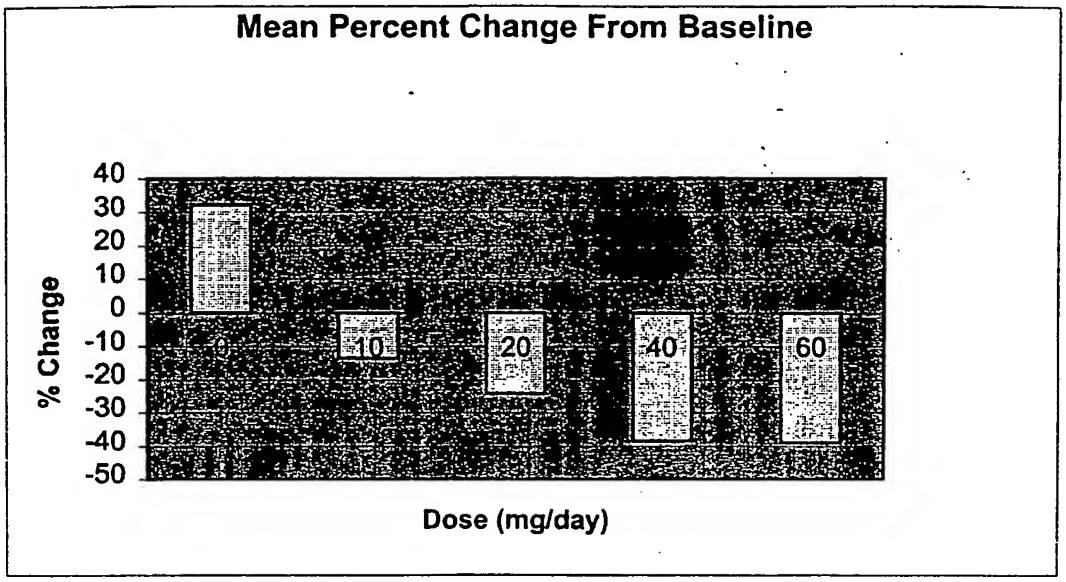
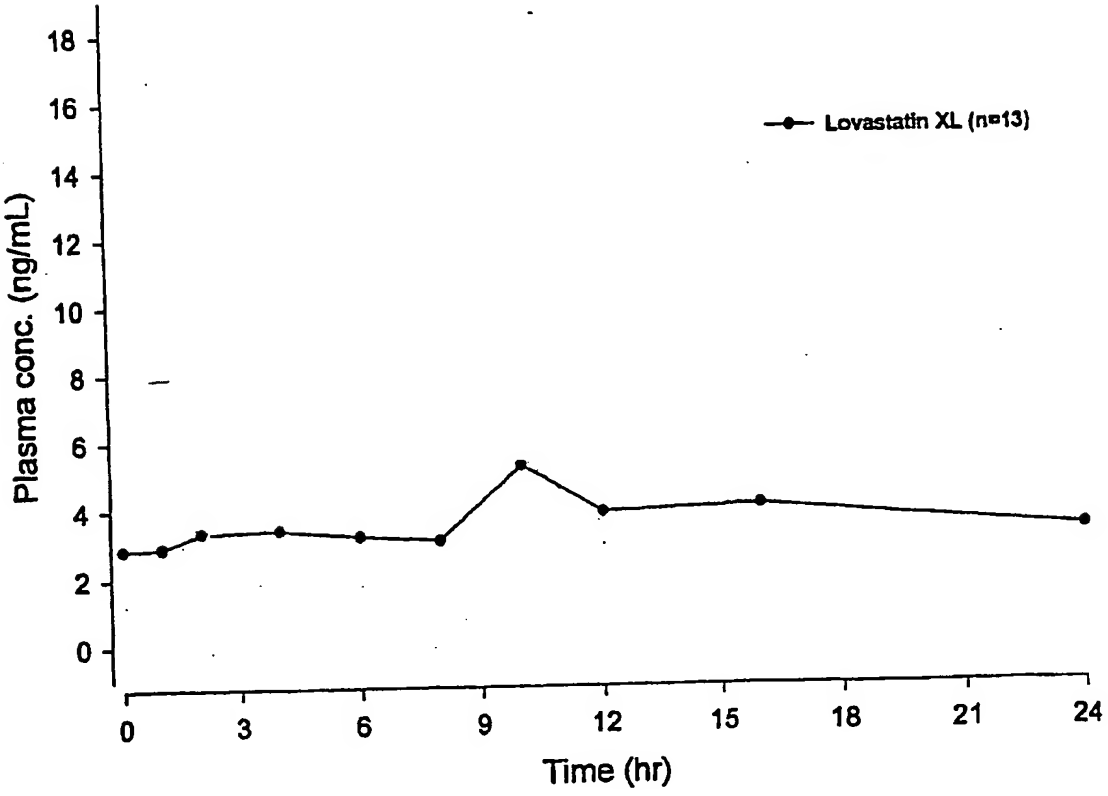

Mean Percent Change From Baseline
% Change
40
30
20
10
0
-10
-20
-30
-40
-50
10
20
40
60
Dose (mg/day)

Figure 7

Mean (±SD) plasma concentration profiles of β-hydroxyacid of lovastatin in patients after multiple oral doses (four weeks) of lovastatin XL



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

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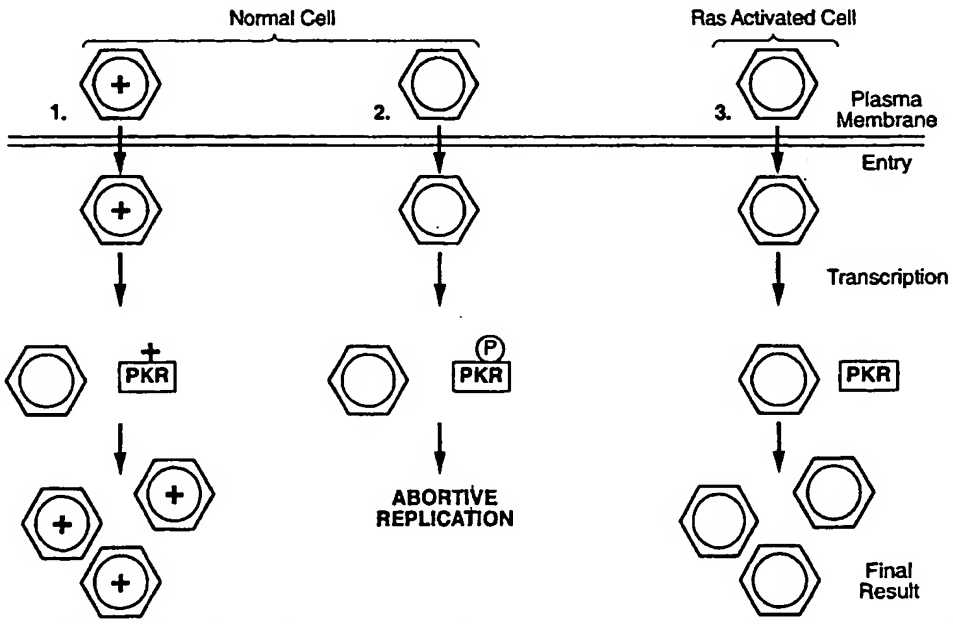
(54) Title: VIRUSES FOR THE TREATMENT OF CELLULAR PROLIFERATIVE DISORDERS



(57) Abstract: Methods for treating cell proliferative disorders by administering virus to proliferative cells having an activated Ras-pathway are disclosed. The virus is administered so that it ultimately directly contacts proliferating cells having an activated Ras-pathway. Proliferative disorders include but are not limited to neoplasms. The virus is selected from modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus. Also disclosed are methods for treating cell proliferative disorders by further administering an immunosuppressive agent.

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VIRUSES FOR THE TREATMENT OF CELLULAR PROLIFERATIVE DISORDERS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of United States Provisional Application Serial Number 60/164,878, filed November 12, 1999, which is incorporated by reference in its entirety.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention pertains to methods for treating cellular proliferative disorders in a mammal that are mediated by Ras-activation using mutant viruses.

References

The following publications, patent applications and patents are cited in this application:

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All of the above publications, patent applications and patents are herein incorporated by reference in their entirety to the same extent as if each individual

publication, patent application or patent was specifically and individually indicated to be incorporated by reference in its entirety.

State of the Art

Normal cell proliferation is regulated by a balance between growth-promoting proto-oncogenes and growth-constraining tumor-suppressor genes. Tumorigenesis can be caused by genetic alterations to the genome that result in the mutation of those cellular elements that govern the interpretation of cellular signals, such as potentiation of proto-oncogene activity or inactivation of tumor suppression. It is believed that the interpretation of these signals ultimately influences the growth and differentiation of a cell, and that misinterpretation of these signals can result in neoplastic growth (neoplasia).

Genetic alteration of the proto-oncogene Ras is believed to contribute to approximately 30% of all human tumors.[18, 19] The role that Ras plays in the pathogenesis of human tumors is specific to the type of tumor. Activating mutations in Ras itself are found in most types of human malignancies, and are highly represented in pancreatic cancer (80%), sporadic colorectal carcinomas (40-50%), human lung adenocarcinomas (15-24%), thyroid tumors (50%) and myeloid leukemia (30%). [20, 21, 22] Ras activation is also demonstrated by upstream mitogenic signaling elements, notably by tyrosine receptor kinases (RTKs). These upstream elements, if amplified or overexpressed, ultimately result in elevated Ras activity by the signal transduction activity of Ras. Examples of this include overexpression of PDGFR in certain forms of glioblastomas, as well as in c-erbB-2/neu in breast cancer.[22, 23, 24]

Protein kinase R ("PKR") is a serine/threonine kinase that is induced in the presence of interferon.[7, 9, 17] The primary cellular substrate of this kinase is the α subunit of the translation initiation factor eIF-2 on Serine 5.[14, 15, 17]

Phosphorylation of eIF-2 results in a rapid inhibition of protein synthesis by preventing its participation in further rounds of translation initiation.

Although PKR is normally inactive, it becomes rapidly activated in the presence of double stranded RNA (dsRNA) or RNAs that exhibit extensive secondary structures, elements that are frequently produced as the result of viral infection. The amino-terminal of PKR contains a double stranded RNA binding domain (dsRBD) that allows this interaction with dsRNA. Binding of PKR to dsRNA element allows PKR to undergo a conformational change that facilitates autophosphorylation and subsequent phosphorylation of eIF-2.[4] Further, it appears that the cooperative binding of two PKR molecules to one dsRNA molecule is required to achieve activation since the addition of dsRNA to PKR results in the dsRNA/PKR activation complex to be found in a 2:1 ratio of protein to dsRNA.[17]

Double-stranded RNA (dsRNA) viruses are not entirely susceptible to the host cell PKR because they have evolved a number of different strategies to inhibit PKR activation in response to their presence:

(1) In the case of adenovirus, a viral product, VAI RNA, is synthesized in large amounts. These VAI RNA elements, with their extensive secondary structure and short length inactivate PKR by acting as a competitive inhibitor of the full length viral dsRNA.[8] The short length of the VAI RNA elements is critical, as there is a minimum length dsRNA which activates PKR. PKR bound to VAI RNA is not activated;

(2) Vaccinia virus encodes two gene products, K3L and E3L to down-regulate PKR with different mechanisms. The K3L gene product has limited homology with the N-terminal region of eIF-2α and may act as a pseudosubstrate for PKR.[1,5] The E3L gene product is a dsRNA-binding protein and apparently functions by sequestering activator dsRNAs;[3,6]

(3) Herpes simplex virus (HSV) gene $_{\gamma_1}$34.5 encodes the gene product infected-cell protein 34.5 (ICP34.5) that can prevent the antiviral effects exerted by PKR; and

(4) The parapoxvirus orf virus encodes the gene OV20.0L that is involved in blocking PKR activity.[30]

It has been demonstrated that in Ras transformed cells, dsRNA-mediated activation of PKR was blocked at the level of autophospohrylation.[16]

PKR is one of many cellular proteins that is induced in the presence of interferon ("IFN"). In normal cells, PKR is normally induced and activated in the presence of IFN. In Ras-mediated tumor cells, however, PKR is induced in the presence of IFN but the activation of PKR is reversed or inhibited. Accordingly, Ras-mediated tumors are unable to activate a PKR response.

It has been observed that pre-treating cells with IFN to induce the transcription and translation of PKR prevents reovirus infection. PKR was activated in cells that were pre-treated with IFN, suggesting that there may be a "quantity effect." When the cells were not pre-treated with IFN, reovirus was able to replicate quickly enough such that there was not enough time to allow sufficient PKR to be synthesized. Additionally, the PKR already present in the cell was not activated. This observation suggests that the cells are not deficient in the IFN response *per se*, since PKR is only one element of the IFN response and PKR apparently acted normally if the cells were pre-treated.

Current methods of treatment for neoplasia include surgery, chemotherapy and radiation. Surgery is typically used as the primary treatment for early stages of cancer; however, many tumors cannot be completely removed by surgical means. In addition, metastatic growth of neoplasms may prevent complete cure of

cancer by surgery. Chemotherapy involves administration of compounds having antitumor activity, such as alkylating agents, antimetabolites, and antitumor antibiotics. The efficacy of chemotherapy is often limited by severe side effects, including nausea and vomiting, bone marrow depression, renal damage, and central nervous system depression. Radiation therapy relies on the greater ability of normal cells, in contrast with neoplastic cells, to repair themselves after treatment with radiation. Radiotherapy cannot be used to treat many neoplasms, however, because of the sensitivity of tissue surrounding the tumor. In addition, certain tumors have demonstrated resistance to radiotherapy and such may be dependent on oncogene or anti-oncogene status of the cell.[25, 26, 27] Martuza et al., EP 0 514 603[32], generically describes methods for selectively killing neoplastic cells which utilize altered viruses that are capable of replication in neoplastic cells while sparing surrounding normal tissue.

Accordingly, it has been found that viruses which have evolved certain mechanisms of preventing PKR activation are likely rendered replication incompetent when these same mechanisms are prevented or mutated. Mutation or deletion of the genes responsible for antagonizing PKR should prevent viral replication in cells in which the PKR activity is normal (i.e. normal cells). However, if infected cells are unable to activate the antiviral response mediated through PKR (i.e., Ras-mediated tumor cells), then these mutant viruses should replicate unheeded and cause cell death. Therefore, these mutant viruses can replicate preferentially in Ras-transformed cells where it is determined that PKR is unable to function.

In view of the drawbacks associated with the current means for treating neoplastic growth, the need still exists for improved methods for the treatment of most types of cancers.

SUMMARY OF THE INVENTION

This invention is directed to a method for treating a Ras-mediated cell proliferative disorder in a mammal, comprising administering to proliferating cells in a mammal having a Ras-activated pathway an effective amount of one or more viruses selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells.

The virus is attenuated or modified such that modified adenovirus comprises a mutant gene encoding VAI RNA, the modified HSV comprises a mutation in the gene $_{\gamma 1}$34.5, the modified vaccinia virus comprises a mutant gene selected from the group consisting of E3L and K3L, and the modified parapoxvirus orf virus comprises a mutation in the OV20.0L gene.

The virus may be modified such that the virion is packaged in a liposome or micelle, or the proteins of the outer capsid have been mutated. The virus can be administered in a single dose or in multiple doses. The cell proliferative disorder may be a neoplasm. Both solid and hematopoietic neoplasms can be targeted.

Also provided is a method of treating a neoplasm having an activated Ras-pathway in a human, comprising administering to the neoplasm an effective amount of virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to result in substantial oncolysis of the neoplastic cells.

The virus may be administered by injection into or near a solid neoplasm.

Also provided is a method of inhibiting metastasis of a neoplasm having an activated Ras-pathway in a mammal, comprising administering to the neoplastic cells in a mammal a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, in an amount sufficient to result in substantial lysis of the neoplasm.

Also provided is a method of treating a neoplasm suspected of having an activated Ras-pathway in a mammal, comprising surgical removal of the substantially all of the neoplasm and administration of a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplasm.

Also provided is a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, a chemotherapeutic agent and a pharmaceutically acceptable excipient.

Also provided is a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a pharmaceutically acceptable excipient.

Further, this invention includes a kit comprising a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a chemotherapeutic agent.

Additionally, this invention provides a kit comprising a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus and an anti-antivirus antibody.

Also provided is a method for treating a population of cells comprising a neoplasm suspected of having an activated Ras-pathway *in vitro* comprising administering to said population of cells *in vitro* a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in an amount sufficient to result in substantial lysis of the neoplasm.

The invention is also directed to methods of treating a Ras-mediated proliferative disorder in a mammal, by immunosuppressing, immunoinhibiting or otherwise rendering the mammal immunodeficient and, concurrently or subsequently, administering a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus in an amount sufficient to result in substantial lysis of the neoplasm. In particular, it is directed to method for treating a Ras-mediated proliferative disorder in a mammal, by a) performing a step selected from the group consisting of:

i) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;

ii) removing B-cells or T-cells from said mammal;

iii) removing anti-virus antibodies from said mammal;

iv) removing antibodies from said mammal;

v) administering anti-antivirus antibodies to said mammal; and

vi) suppressing the immune system of the mammal; and

b) administering to the proliferating cells in said mammal an effective amount of one or more viruses selected from the group consisting of modified adenovirus,

modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells.

The methods and pharmaceutical compositions of the invention provide an effective means to treat neoplasia having an activated Ras-pathway, without the side effects associated with other forms of cancer therapy.

The foregoing and other objects, features and advantages of the invention will be apparent from the following more particular description of preferred embodiments of the invention, as illustrated in the accompanying figure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a depiction of the molecular basis of VAI defective adenovirus oncolysis.

DETAILED DESCRIPTION OF THE INVENTION

The invention pertains to methods of treating a Ras-mediated proliferative disorder in a mammal, by administering a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to the proliferating cells.

Definitions

The following terms used herein are defined as follows:

"Adenovirus" is a double stranded DNA virus of about 3.6 kilobases. In humans, adenoviruses can replicate and cause disease in the eye and in the

respiratory, gastrointestinal and urinary tracts. About one-third of the 47 known human serotypes are responsible for most cases of human adenovirus disease.[28] The adenovirus encodes several gene products that counter antiviral host defense mechanisms. The virus-associated RNA (VAI RNA or VA RNA_I) of the adenovirus are small, structured RNAs that accumulate in high concentrations in the cytoplasm at late time after adenovirus infection. These VAI RNA bind to the to the double stranded RNA (dsRNA) binding motifs of PKR and block the dsRNA-dependent activation of PKR by autophosphorylation. Thus, PKR is not able to function and the virus can replicate within the cell. The overproduction of virons eventually leads to cell death. The attenuated or modified adenovirus is unable to replicate in cells which do not have an activated Ras-pathway. However, attenuated or modified adenovirus can replicate in cells with an activated Ras-pathway.

The term "attenuated adenovirus" or "modified adenovirus" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the VAI RNA's are not transcribed. Such attenuated or modified adenovirus would not be able to replicate in normal cells that do not have an activated Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

"Herpes simplex virus" (HSV) refers to herpes simplex virus-1 (HSV-1) or herpes simplex virus-2 (HSV-2). HSV gene $_{\gamma 1}34.5$ encodes the gene product infected-cell protein 34.5 (ICP34.5) that can prevent the antiviral effects exerted by PKR. ICP34.5 has a unique mechanism of preventing PKR activity by interacting with protein phosphatase 1 and redirecting it activity to dephosphorylate eIF-2α.[29] In cells infected with either wild-type or the genetically engineered virus from which the $_{\gamma 1}34.5$ genes were deleted, eIF-2α is

phosphorylated and protein synthesis is turned off in cells infected with $_{\gamma 1}$34.5 minus virus. It would be expected that the $_{\gamma 1}$34.5 minus virus would be replication competent in cells with an activated Ras pathway in which the activity of ICP34.5 would be redundant. HSV is unable to replicate in cells which do not have an activated Ras-pathway. Thus, HSV can replicate in cells which have an activated Ras-pathway.

The term "attenuated HSV" or "modified HSV" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the HSV gene $_{\gamma 1}$34.5 is not transcribed. Such attenuated or modified HSV would not be able to replicate in normal cells that do not have an activated Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

"Parapoxvirus Orf Virus" is a poxvirus. It is a virus that induces acute cutaneous lesions in different mammalian species, including humans. Parapoxvirus orf virus naturally infects sheep, goats and humans through broken or damaged skin, replicates in regenerating epidermal cells and induces pustular leasions that turn to scabs.[30] The parapoxvirus orf virus encodes the gene OV20.0L that is involved in blocking PKR activity.[30] The parapoxvirus orf virus is unable to replicate in cells which do not have an activated Ras-pathway. Thus, the parapoxvirus orf virus replicate in cells which have an activated Ras-pathway.

The term "attenuated parapoxvirus orf virus" or "modified parapoxvirus orf virus" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the gene OV20.0L is not transcribed. Such attenuated or modified parapoxvirus orf virus would not be able to replicate in normal cells that do not

have an activated Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

"Vaccinia virus" refers to the virus of the orthopoxvirus genus that infects humans and produces localized lesions.[28] Vaccinia virus encodes two genes that play a role in the down regulation of PKR activity through two entirely different mechanisms. E3L gene encodes two proteins of 20 and 25 kDa that are expressed early in infection and have dsRNA binding activity that can inhibit PKR activity. Deletion or disruption of the E3L gene creates permissive viral replication in cells having an activated Ras pathway. The K3L gene of vaccinia virus encodes pK3, a pseudosubstrate of PKR.

Deletion of residues which disrupt E3 function to inhibit the dsRNA binding. Additionally, since the amino terminal region of E3 protein interacts with the carboxy-terminal region domain of PKR, deletion or point mutation of this domain prevents anti-PKR function. Chang et al., *PNAS* 89:4825-4829 (1992); Chang et al., *Virol.* 194:537-547 (1993); Chang et al. *J. Virol.* 69:6605-6608 (1995); Sharp et al. *Virol.* 250:302-315 (1998); and Romano et al., *Molecular and Cellular Bio.*, 18(12):7304-7316 (1998). The K3L gene of vaccinia virus encodes pK3, a pseudosubstrate of PKR. There is a loss-of-function mutation within K3L. By either truncating or by placing point mutations within the C-terminal portion of K3L protein, homologous to residues 79 to 83 in eIF-2α abolish PKR inhibitory activity.[31]

The term "attenuated vaccinia virus" or "modified vaccinia virus" means that the gene product or products which prevent the activation of PKR are lacking, inhibited or mutated such that PKR activation is not blocked. Preferably, the E3L gene and/or the K3L gene is not transcribed. Such attenuated or modified vaccinia virus would not be able to replicate in normal cells that do not have an activated

Ras-pathway, however, it would be able to infect and replicate in cells having an activated Ras-pathway.

A "proliferative disorder" is any cellular disorder in which the cells proliferate more rapidly than normal tissue growth. Thus a "proliferating cell" is a cell that is proliferating more rapidly than normal cells. The proliferative disorder, includes but is not limited to neoplasms. A "neoplasm" is an abnormal tissue growth, generally forming a distinct mass, that grows by cellular proliferation more rapidly than normal tissue growth. Neoplasms show partial or total lack of structural organization and functional coordination with normal tissue. These can be broadly classified into three major types. Malignant neoplasms arising from epithelial structures are called carcinomas, malignant neoplasms that originate from connective tissues such as muscle, cartilage, fat or bone are called sarcomas and malignant tumors affecting hematopoetic structures (structures pertaining to the formation of blood cells) including components of the immune system, are called leukemias and lymphomas. A tumor is the neoplastic growth of the disease cancer. As used herein, a neoplasm, also referred to as a "tumor", is intended to encompass hematopoietic neoplasms as well as solid neoplasms. Other proliferative disorders include, but are not limited to neurofibromatosis.

"Administration to a proliferating cell or neoplasm" indicates that the virus is administered in a manner so that it contacts the proliferating cells or cells of the neoplasm (also referred to herein as "neoplastic cells").

A "mammal suspected of having a proliferative disorder" means that the mammal may have a proliferative disorder or tumor or has been diagnosed with a proliferative disorder or tumor or has been previously diagnosed with a proliferative disorder or tumor, the tumor or substantially all of the tumor has

been surgically removed and the mammal is suspected of harboring some residual tumor cells.

"Viral infection" or "virus infection" as used herein refers to infection by one or more of adenovirus, HSV, parapoxvirus orf virus, or vaccinia virus.

"Resistance" of cells to viral infection indicates that infection of the cells with the virus does not result in significant viral production or yield. Without being limited to any theory, resistance to viral infection is believed to be found at the level of gene translation, rather than at early transcription. While viral transcripts are produced, viral proteins are not expressed. It is thought that viral gene transcription in resistant cells correlated with phosphorylation of an approximately 65 kDa cell protein, determined to be double-stranded RNA-activated protein kinase (PKR), that was not observed in transformed cells. Phosphorylation of PKR lead to inhibition of translation.

The term "substantial lysis" means at least 10% of the proliferating cells are lysed, more preferably of at least 50% and most preferably of at least 75% of the cells are lysed. The percentage of lysis can be determined for tumor cells by measuring the reduction in the size of the tumor in the mammal or the lysis of the tumor cells *in vitro*.

"Anti-virus antibody" refers to an antibody which binds to a particular virus. For example, an anti-virus antibody may be an anti-adenovirus antibody, an anti-HSV antibody, an anti-vaccinia virus antibody or an anti-parapoxvirus orf virus antibody. The particular anti-virus antibody selected for use in the methods of this invention will correspond to the virus which is administered to the patient. For example, an anti-HSV antibody would be used in the method where a modified HSV is administered.

"Anti-antivirus antibodies," are antibodies directed against anti-virus antibodies. Anti-antivirus antibodies used in this invention are selected from anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies. Such antibodies can be made by methods known in the art. See for example "Antibodies: A laboratory manual" E. Harlow and D. Lane, Cold Spring Harbor Laboratory (1988).

"IgG antibodies" refers to immunoglobulin G antibodies. IgG, the most abundant type of antibody, carries the major burden of neutralizing bacterial toxins and binding to microorganisms to enhance their phagocytosis.

"Humanized antibodies" refers to antibody molecules in which the amino acid sequence in the non-antigen binding regions has been altered so that the antibody more closely resembles a human antibody, and still retains its original binding ability.

The terms "immunosuppressant" or "immune suppressive agent" include conventional immunosuppressants, immunoinhibitors, antibodies, and conditions such as radiation therapy or HIV infection which result in compromise of the immune system.

"B-cells" refers to B-lymphocytes. There are two major subpopulations of B lymphocytes, B-1 and B-2 cells. B-1 cells are self-renewing and frequently secrete high levels of antibody which bind to a range of antigens (polyspecificity) with a relatively low affinity. The majority of B cells, B-2 cells, are directly generated from precursors in the bone marrow and secrete highly specific antibody.

"T-cells" refers to T-lymphocytes. T-cells differentiate within the thymus gland and are specialized to operate against cells bearing intracellular organisms. T-cells only recognize antigen when it is on the surface of a body cell.

It is believed that the virus uses the host cell's Ras pathway machinery to downregulate PKR and thus reproduce. Figure 1 depicts the usurpation of the host cell Ras signalling pathway by adenovirus. As shown in Figure 1, in both untransformed and Ras-activated cells, wild-type adenovirus (denoted with +) and VAI defective adenovirus (open circle) are both able to bind, internalize and undergo early transcription in a normal fashion.

During transcription, wild-type adenovirus (panel #1) is able to transcribe VAI RNAs that can bind to PKR without activating it. Because PKR is unable to displace these short, double stranded RNAs (dsRNAs), PKR is unable to interact with subsequent longer transcripts and autophosphorylate. Thus, the virus is able to replicate and produce progeny virus.

When attempting to replicate in untransformed cells (panel #2), modified adenovirus is unable to produce the VAI RNAs which bind to PKR. Thus, PKR can interact with longer viral transcripts that are capable of causing autophosphorylation and activate PKR. The activated PKR is then able to phosphorylate the translation initiation factor eIF-2α and block translation of viral genes that lead to abortive viral replication.

Panel #3 shows the modified adenovirus infecting a Ras-activated cancer cell where the outcome is different from the outcome described in panels #1 and #2. In the Ras-transformed cells, it has been observed that PKR is unable to undergo phosphorylation or that phosphorylaion is rapidly reversed by an element of the activated Ras pathway. The result in the Ras-activated cells is that the

modified form of the adenovirus is able to translate its viral genes and complete replication without the transcription of the VAI RNAs. The surprising result in these cells is oncolysis.

As is known in the art, the implantation of human tumor cells into SCID mice is recognized as a well known model system for testing the effectiveness of various anti-tumor agents in humans. It has previously been shown that pharmaceuticals effective against human tumors implanted into SCID mice can be predictive of their effectiveness against the same tumors in humans.

Based upon these discoveries, Applicants have developed methods for treating cell proliferative disorders in mammals wherein the cells have an activated Ras-pathway. Representative mammals include dogs, cats, sheep, goats, cattle, horses, pigs, non-human primates, and humans. In a preferred embodiment, the mammal is a human.

Methods of the Invention

In the methods of the invention, modified virus is administered to proliferating cells having an activated Ras-pathway in the individual mammal. Representative types of modified virus include adenovirus, HSV, parapoxvirus orf virus, or vaccinia virus which infect humans. In a preferred embodiment, modified adenovirus is used.

The virus may be a recombinant virus from two or more types of viruses with differing pathogenic phenotypes such that it contains different antigenic determinants thereby reducing or preventing an immune response by a mammal previously exposed to a virus subtype. Such recombinant virions can be generated by co-infection of mammalian cells with different subtypes of virus with the

resulting resorting and incorporation of different subtype coat proteins into the resulting virion capsids.

The virus may be modified by incorporation of mutated coat proteins into the virion outer capsid. The proteins may be mutated by replacement, insertion or deletion. "Replacement" includes the insertion of different amino acids in place of the native amino acids. "Insertions" include the insertion of additional amino acid residues into the protein at one or more locations. "Deletions" include deletions of one or more amino acid residues in the protein. Such mutations may be generated by methods known in the art. For example, oligonucleotide site directed mutagenesis of the gene encoding for one of the coat proteins could result in the generation of the desired mutant coat protein. Expression of the mutated protein in virus infected mammalian cells *in vitro* such as COS1 cells will result in the incorporation of the mutated protein into the virus virion particle

The virus is preferably a virus modified to reduce or eliminate an immune reaction to the virus. Such modified virus are termed "immunoprotected virus". Such modifications could include packaging of the virus in a liposome, a micelle or other vehicle to mask the virus from the mammals immune system.

At least some of the cells of the proliferative disorder have a mutation in which the Ras gene (or an element of the Ras signaling pathway) is activated, either directly (e.g., by an activating mutation in Ras) or indirectly (e.g., by activation of an upstream element in the Ras pathway). Activation of an upstream element in the Ras pathway includes, for example, transformation with epidermal growth factor receptor (EGFR) or Sos. A proliferative disorder that results, at least in part, by the activation of Ras, an upstream element of Ras, or an element in the Ras signalling pathway is referred to herein as a "Ras-mediated proliferative disorder".

One neoplasm that is particularly susceptible to treatment by the methods of the invention is pancreatic cancer, because of the prevalence of Ras-mediated neoplasms associated with pancreatic cancer. Other neoplasms that are particularly susceptible to treatment by the methods of the invention include breast cancer, central nervous system cancer (e.g., neuroblastoma and glioblastoma), peripheral nervous system cancer, lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, lymphoma and leukemia. One proliferative disorder that is particularly susceptible to treatment by the methods of this invention include neurofibromatosis 1 because of the activation of the Ras pathway.

The virus is administered to a proliferating cell or neoplasm in a manner so that it contacts the proliferating cells or cells of the neoplasm or neoplastic cells. The route by which the virus is administered, as well as the formulation, carrier or vehicle, will depend on the location as well as the type of the neoplasm. A wide variety of administration routes can be employed. For example, for a solid neoplasm that is accessible, the virus can be administered by injection directly to the neoplasm. For a hematopoietic neoplasm, for example, the virus can be administered intravenously or intravascularly. For neoplasms that are not easily accessible within the body, such as metastases or brain tumors, the virus is administered in a manner such that it can be transported systemically through the body of the mammal and thereby reach the neoplasm (e.g., intrathecally, intravenously or intramuscularly).

Alternatively, the virus can be administered directly to a single solid neoplasm, where it then is carried systemically through the body to metastases. The virus can also be administered subcutaneously, intraperitoneally, topically (e.g., for melanoma), orally (e.g., for oral or esophageal neoplasm), rectally (e.g.,

for colorectal neoplasm), vaginally (e.g., for cervical or vaginal neoplasm), nasally or by inhalation spray (e.g., for lung neoplasm).

Virus can be administered systemically to mammals which are immune compromised or which have not developed immunity to the virus epitopes. In such cases, virus administered systemically, i.e. by intraveneous injection, will contact the proliferating cells resulting in lysis of the cells.

Immunocompetent mammals previously exposed to a particular virus, such as modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, may have developed humoral and/or cellular immunity to that virus. Nevertheless, it is contemplated that direct injection of the virus into a solid tumor in immunocompetent mammals will result in the lysis of the neoplastic cells.

When the virus is administered systemically to immunocompetent mammals, the mammals may produce an immune response to the virus. Such an immune response may be avoided if the virus is of a subtype to which the mammal has not developed immunity, or the virus has been modified as previously described herein such that it is immunoprotected, for example, by protease digestion of the outer capsid or packaging in a micelle.

It is contemplated that the virus may be administered to immunocompetent mammals immunized against the virus in conjunction with the administration of anti-antivirus antibodies. Such anti-antivirus antibodies may be administered prior to, at the same time or shortly after the administration of the virus. Preferably an effective amount of the anti-antivirus antibodies are administered in sufficient time to reduce or eliminate an immune response by the mammal to the administered virus.

Alternatively, it is contemplated that the immunocompetency of the mammal against the virus may be suppressed either by the prior or co-administration of pharmaceuticals known in the art to suppress the immune system in general (Cuff et al., "Enteric reovirus infection as a probe to study immunotoxicity of the gastrointestinal tract" *Toxicological Sciences* 42(2):99-108 (1998)) or alternatively the administration of such immunoinhibitors as anti-antivirus antibodies.

The humoral immunity of the mammal against the virus may also be temporarily reduced or suppressed by plasmaphoresis of the mammals blood to remove the anti-virus antibodies. The anti-virus antibodies removed by this process correspond to the virus selected for administration to the patient. For example, if a modified parapox orf virus is selected for administration, then the anti-parapox orf viruse antibodies will be removed. The humoral immunity of the mammal against the virus may additionally be temporarily reduced or suppressed by the intraveneous administration of non-specific immunoglobulin to the mammal.

Other agents are known to have immunosuppressant properties as well (see, e.g., Goodman and Gilman, 7th Edition, page 1242, the disclosure of which is incorporated herein by reference). Such immunoinhibitors also include anti-antivirus antibodies, which are antibodies directed against anti-virus antibodies. Anti-antivirus antibodies used in this invention are selected from anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies. Such antibodies can be made by methods known in the art. See for example "Antibodies: A laboratory manual" E. Harlow and D. Lane, Cold Spring Harbor Laboratory (1988).

Such anti-antivirus antibodies may be administered prior to, at the same time or shortly after the administration of the virus. Preferably an effective amount of the anti-antivirus antibodies are administered in sufficient time to reduce or eliminate an immune response by the mammal to the administered virus.

In yet other methods of the invention, a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus is administered to Ras-mediated proliferating cells in the individual mammal. In one embodiment of this invention a course of this therapy is administered one or more times. Following the first administration of virus therapy particular immune constituents that may interfere with subsequent administrations of virus are removed from the patient. These immune constituents include B cells, T cells, antibodies, and the like.

Removal of either the B cell or T cell population can be accomplished by several methods. In one method, the blood may be filtered and heme-dialysis may be performed. Another method is the filtration of the blood coupled with extra corporeal compounds that can remove the cell populations, for example, with immobilized antibodies that recognize specific receptors on the cell population which is to be remove. Yet another method for removal of a cell population is by immune suppression. This can be done by first line radiation therapy or by cyclic steroids such as cyclosporin.

Selective removal of anti-virus antibodies can also prevent the patient's immune system from removing therapeutically administered virus. Preventing antibody interaction with the administered virus may also assist systemic treatment strategies. Antibodies can be removed by several methods, including heme-dialysis and passing the blood over immobilized virus (selective antibody removal); by removal of all IgG antibodies by heme-dialysis and passing the blood

over immobilized protein A (commercially available as PROSORBA, Cypress Bioscience, San Diego, CA); or by administration of humanized anti-idiotypic antibodies, where the idiotype is against the virus to be administered (e.g., a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus).

Another method of this invention is to allow virus to act systemically without impairing normal immune function by masking or impairing immune recognition of virus. To prevent the patient's immune system from recognizing the administered virus, the virus may be coated with non-virotoxic humanized antibodies, such as coating with the F_{ab} portion of the antibody, or coated in a micelle.

Additionally, the virus may be treated with chymotrypsin to yield an infectious subviral particle (ISVP). An ISVP may be used either alone or in combination with whole virus to provide an agent that is either poorly recognized has not been previously prevented by the patient's immune system.

Another embodiment of this invention includes the removal of virus from the patient following administration. Since this method may be used on patients that are either immune suppressed or immune incompetent, it may be of importance to remove virus from the blood stream following the course of treatment. virus may be removed by affinity chromatography using extra corporeal anti-virus antibodies associated with heme dialysis, B-cell proliferative agents, or adjuvants to stimulate immune response against the virus such as UV inactivated virus or Freund's adjuvant.

Pharmaceutical Compositions

This invention also includes pharmaceutical compositions which contain, as the active ingredient, one or more of the viruses associated with "pharmaceutically acceptable carriers or excipients." This invention also includes pharmaceutical compositions which contain, as the active ingredient, one or more immunosuppressants or immunoinhibitors and one or more of the viruses associated with "pharmaceutically acceptable carriers or excipients."

In making the compositions of this invention, the active ingredient(s), e.g., the virus and/or immunosuppressant or immunoinhibitor, are usually mixed with an excipient, diluted by an excipient or enclosed within such a carrier which can be in the form of a capsule, sachet, paper or other container.

When the pharmaceutically acceptable excipient serves as a diluent, it can be a solid, semi-solid, or liquid material, which acts as a vehicle, carrier or medium for the active ingredient. Thus, the compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments containing, for example, up to 10% by weight of the active compound, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders.

Some examples of suitable excipients include lactose, dextrose, sucrose, sorbitol, mannitol, starches, gum acacia, calcium phosphate, alginates, tragacanth, gelatin, calcium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, sterile water, syrup, and methyl cellulose. The formulations can additionally include: lubricating agents such as talc, magnesium stearate, and mineral oil; wetting agents; emulsifying and suspending agents; preserving agents such as methyl- and propylhydroxy-benzoates; sweetening agents; and flavoring

agents. The compositions of the invention can be formulated so as to provide quick, sustained or delayed release of the active ingredient after administration to the patient by employing procedures known in the art.

For preparing solid compositions such as tablets, the principal active ingredient/virus is mixed with a pharmaceutical excipient to form a solid preformulation composition containing a homogeneous mixture of a compound of the present invention. When referring to these preformulation compositions as homogeneous, it is meant that the active ingredient is dispersed evenly throughout the composition so that the composition may be readily subdivided into equally effective unit dosage forms such as tablets, pills and capsules.

The tablets or pills of the present invention may be coated or otherwise compounded to provide a dosage form affording the advantage of prolonged action. For example, the tablet or pill can comprise an inner dosage and an outer dosage component, the latter being in the form of an envelope over the former. The two components can be separated by an enteric layer which serves to resist disintegration in the stomach and permit the inner component to pass intact into the duodenum or to be delayed in release. A variety of materials can be used for such enteric layers or coatings, such materials including a number of polymeric acids and mixtures of polymeric acids with such materials as shellac, cetyl alcohol, and cellulose acetate.

The liquid forms in which the novel compositions of the present invention may be incorporated for administration orally or by injection include aqueous solutions, suitably flavored syrups, aqueous or oil suspensions, and flavored emulsions with edible oils such as corn oil, cottonseed oil, sesame oil, coconut oil, or peanut oil, as well as elixirs and similar pharmaceutical vehicles.

Compositions for inhalation or insufflation include solutions and suspensions in pharmaceutically acceptable, aqueous or organic solvents, or mixtures thereof, and powders. The liquid or solid compositions may contain suitable pharmaceutically acceptable excipients as described herein. Preferably the compositions are administered by the oral or nasal respiratory route for local or systemic effect. Compositions in preferably pharmaceutically acceptable solvents may be nebulized by use of inert gases. Nebulized solutions may be inhaled directly from the nebulizing device or the nebulizing device may be attached to a face mask tent, or intermittent positive pressure breathing machine. Solution, suspension, or powder compositions may be administered, preferably orally or nasally, from devices which deliver the formulation in an appropriate manner.

Another preferred formulation employed in the methods of the present invention employs transdermal delivery devices ("patches"). Such transdermal patches may be used to provide continuous or discontinuous infusion of the virus of the present invention in controlled amounts. The construction and use of transdermal patches for the delivery of pharmaceutical agents is well known in the art. See, for example, U.S. Patent 5,023,252, herein incorporated by reference. Such patches may be constructed for continuous, pulsatile, or on demand delivery of pharmaceutical agents.

Other suitable formulations for use in the present invention can be found in *Remington's Pharmaceutical Sciences*.

Kits of Parts

The virus or the pharmaceutical composition comprising the virus may be packaged into convenient kits providing the necessary materials packaged into suitable containers. It is contemplated the kits may also include chemotherapeutic agents and/or anti-antivirus antibody.

The immunosuppressant or immunoinhibitor and virus or the pharmaceutical composition comprising the immunosuppressant or immunoinhibitor and virus may be packaged into convenient kits providing the necessary materials packaged into suitable containers. It is contemplated the kits may also include chemotherapeutic agent.

Administration of Virus

The virus is administered in an amount that is sufficient to treat the proliferative disorder (e.g., an "effective amount"). A proliferative disorder is "treated" when administration of virus to the proliferating cells effects lysis of the proliferating cells. This may result in a reduction in size of the neoplasm, or in a complete elimination of the neoplasm. The reduction in size of the neoplasm, or elimination of the neoplasm, is generally caused by lysis of neoplastic cells ("oncolysis") by the virus.

Preferably, the effective amount is that amount able to inhibit tumor cell growth. Preferably the effective amount is from about 1.0 pfu/kg body weight to about 10^{15} pfu/kg body weight, more preferably from about 10^{2} pfu/kg body weight to about 10^{13} pfu/kg body weight. For example, for treatment of a human, approximately 10^{2} to 10^{17} plaque forming units (PFU) of virus can be used, depending on the type, size and number of tumors present. The effective amount will be determined on an individual basis and may be based, at least in part, on consideration of the type of virus; the chosen route of administration; the individual's size, age, gender; the severity of the patient's symptoms; the size and other characteristics of the neoplasm; and the like. The course of therapy may last from several days to several months or until diminution of the disease is achieved.

The virus can be administered in a single dose, or multiple doses (i.e., more than one dose). The multiple doses can be administered concurrently, or

consecutively (e.g., over a period of days or weeks). The virus can also be administered to more than one neoplasm in the same individual.

The compositions are preferably formulated in a unit dosage form, each dosage containing from about 10^2 pfus to about 10^{13} pfus of the virus. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of virus calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

It has been found that the virus is effective for the treatment of solid neoplasms in immunocompetent mammals. Administration of unmodified virus directly to the neoplasm results in oncolysis of the neoplastic cells and reduction in the size of the tumor.

It is contemplated that the virus may be administered in conjunction with surgery or removal of the neoplasm. Therefore, provided herewith are methods for the treatment of a solid neoplasm comprising surgical removal of the neoplasm and administration of a virus at or near to the site of the neoplasm.

It is contemplated that the virus may be administered in conjunction with or in addition to radiation therapy.

It is further contemplated that the virus of the present invention may be administered in conjunction with or in addition to known anti-cancer compounds or chemotherapeutic agents. Chemotherapeutic agents are compounds which may inhibit the growth of tumors. Such agents, include, but are not limited to, 5-fluorouracil, mitomycin C, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclins (Epirubicin and Doxurubicin), antibodies

to receptors, such as herceptin, etopside, pregnasome, platinum compounds such as carboplatin and cisplatin, taxanes such as taxol and taxotere, hormone therapies such as tamoxifen and anti-estrogens, interferons, aromatase inhibitors, progestational agents and LHRH analogs. In one embodiment of the invention, a method is provided for reducing the growth of metastastic tumors in a mammal comprising administering an effective amount of a virus to the mammal.

Administration of Virus with Immunosuppressant or Immunoinhibitor

The immunosuppressant or immunoinhibitor is administered in an appropriate amount and using an appropriate schedule of administration sufficient to result in immunosuppression or immunoinhibition of the mammal's immune system. Such amounts and schedules are well known to those of skill in the art.

The immunosuppressant or immunoinhibitor and virus can be administered in a single dose, or multiple doses (i.e., more than one dose). The multiple doses can be administered concurrently, or consecutively (e.g., over a period of days or weeks). The virus can also be administered to more than one neoplasm in the same individual.

The compositions are preferably formulated in a unit dosage form, each dosage containing an appropriate amount of immunosuppressant or immunoinhibitor and from about 10^2 pfus to about 10^{13} pfus of the virus. The term "unit dosage forms" refers to physically discrete units suitable as unitary dosages for human subjects and other mammals, each unit containing a predetermined quantity of virus calculated to produce the desired therapeutic effect, in association with a suitable pharmaceutical excipient.

As mentioned above, it has been found that the virus is effective for the treatment of solid neoplasms in immunocompetent mammals. Administration of

unmodified virus directly to the neoplasm results in oncolysis of the neoplastic cells and reduction in the size of the tumor in immunocompetent animals. When animals are rendered immunosuppressed or immunodeficient in some way, systemic administration of virus will be more effective in producing oncolysis.

It is contemplated that the virus may be administered in conjunction with or in addition to radiation therapy which renders the mammal immunosuppressed. It is further contemplated that the virus and immunosuppressant or immunoinhibitor may be administered in conjunction with or in addition to known anti-cancer compounds or chemotherapeutic agents. Chemotherapeutic agents are compounds which may inhibit the growth of tumors. Such agents, include, but are not limited to, 5-fluorouracil, mitomycin C, methotrexate, hydroxyurea, cyclophosphamide, dacarbazine, mitoxantrone, anthracyclins (Epirubicin and Doxurubicin), antibodies to receptors, such as herceptin, etopside, pregnasome, platinum compounds such as carboplatin and cisplatin, taxanes such as taxol and taxotere, hormone therapies such as tamoxifen and anti-estrogens, interferons, aromatase inhibitors, progestational agents and LHRH analogs.

The virus and immunosuppressants of the present invention are contemplated to reduce the growth of tumors that are metastatic. In an embodiment of the invention, a method is provided for reducing the growth of metastatic tumors in a mammal comprising administering an effective amount of a virus to the immunosuppressed mammal.

It is contemplated that the selected virus may be administered to immunocompetent mammals immunized against the selected virus in conjunction with the administration of immunosuppressants and/or immunoinhibitors. For example, if a modified vaccinia virus is selected then the immunocompetent

mammal is immunized against vaccinia virus. Such immunosuppressants and immunoinhibitors are known to those of skill in the art and include such agents as cyclosporin, rapamycin, tacrolimus, mycophenolic acid, azathioprine and their analogs, and the like.

Utility

The viruses of the present invention may be used for a variety of purposes. They may be used in methods for treating Ras-mediated proliferative disorders in a mammal. The virus may be used to reduce or eliminate neoplasms. They may be used in methods for treating metastases. They may be used in conjunction with known treatments for cancer including surgery, chemotherapy and radiation.

In order to further illustrate the present invention and advantages thereof, the following specific examples are given but are not meant to limit the scope of the claims in any way.

EXAMPLES

In the examples below, all temperatures are in degrees Celsius (unless otherwise indicated) and all percentages are weight percentages (also unless otherwise indicated).

In the examples below, the following abbreviations have the following meanings. If an abbreviation is not defined, it has its generally accepted meaning:

μM	=	micromolar
mM	=	millimolar
M	=	molar
ml	=	milliliter
μl	=	microliter
mg	=	milligram

μg	=	microgram
DNA	=	deoxyribonucleic acid
RNA	=	ribonucleic acid
PAGE	=	polyacrylamide gel electrophoresis
rpm	=	revolutions per minute
FBS	=	fetal bovine serum
DTT	=	dithiothrietol
SDS	=	sodium dodecyl sulfate
PBS	=	phosphate buffered saline
DMEM	=	Dulbecco's modified Eagle's medium
α-MEM	=	α-modified Eagle's medium
β-ME	=	β-mercaptoethanol
MOI	=	multiplicity of infection
PFU	=	plaque forming units
MAPK	=	MAP kinase
phosph-MAPK	=	phosphorylated-MAP kinase
HRP	=	horseradish-peroxidase
PKR	=	double-stranded RNA activated protein kinase
RT-PCR	=	reverse transcriptase-polymerase chain reaction
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase
EGFR	=	epidermal growth factor receptors
MEK kinase	=	mitogen-activated extracellular signal-regulated kinase
DMSO	=	dimethylsulfoxide
SCID	=	severe combined immunodeficiency

General Methods

Cells and Virus

293 cells (human embryonic kidney (HEK) cells (available from ATCC)) are grown as monolayers in Dulbecco's modified Eagle's medium (DMEM, GIBCO Laboratories) supplemented with 10% newborn calf serum (NC) and as suspension cultures in minimal essential medium (SMEM, GIBCO Laboratories) supplemented with 5% NCS.

VAI mutant adenovirus are propagated in suspension cultures of 293 cells maintained in the same medium. Plaque assays are performed on HeLa and 293

monolayers in DMEME containing 0.7% agarose, 2% NCS, 2mM L-glutamine, MEM nonessential acids (GIBCO Laboratories), and 25mM $MgCl_2$.

EXAMPLE 1

In Vivo Oncolytic Capability of Adenovirus Against Human Breast Cancer-Derived Cell Lines

In vivo studies are carried out using human breast carcinoma cells in a SCID mouse model. Female SCID mice are injected with 1 x 10^6 human breast carcinoma MDA-MB468 cells in two subcutaneous sites, overlying both hind flanks. Palpable tumors are evident approximately two to four weeks post injection. Undiluted adenovirus is injected into the right side tumor mass in a volume of 20 μl at a concentration of 1.0 x 10^7 PFU/ml.

EXAMPLE 2

Susceptibility of Additional Human Tumors to Adenovirus Oncolysis

Cells and Virus

All cell lines are grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS).

The adenovirus used in these studies is propagated in suspension cultures of L cells and purified as described above.

Cytopathic effects of adenovirus on cells

Confluent monolayers of cells are infected with adenovirus at a multiplicity of infection (MOI) of approximately 40 plaque forming units (PFU) per cell.

Pictures are taken at 36 hour postinfection for both adenovirus-infected and mock-infected cells.

Immunofluorescent analysis of adenovirus infection

For the immunofluorescent studies the cells are grown on coverslips, and infected with adenovirus at a multiplicity of infection (MOI) of ~10 PFU/cell or mock-infected as described above. At various times postinfection, cells are fixed in an ethanol/acetic acid (20/1) mixture for 5 minutes, then rehydrated by subsequential washes in 75%, 50% and 25% ethanol, followed by 4 washes with phosphate-buffered saline (PBS). The fixed and rehydrated cells are then exposed to the primary antibody (rabbit polyclonal anti-adenovirus serum diluted 1/100 in PBS) for 2 hr at room temperature. Following 3 washes with PBS, the cells are exposed to the secondary antibody [goat anti-rabbit IgG (whole molecule) fluorescein isothiocyanate (FITC) conjugate diluted 1/100 in PBS containing 10% goat serum and 0.005% Evan's Blue counterstain] for 1 hour at room temperature. Finally, the fixed and treated cells are washed 3 more times with PBS, followed by 1 wash with double-distilled water, dried and mounted on slides in 90% glycerol containing 0.1% phenylenediamine, and viewed with a Zeiss Axiophot microscope mounted with a Carl Zeiss camera (magnification for all pictures was 200 x).

Infection of cells and quantitation of virus

Confluent monolayers of cells grown in 24-well plates are infected with adenovirus at an estimated multiplicity of 10 PFU/cell. After 1 hour incubation at 37°C, the monolayers are washed with warm DMEM-10% FBS, and then incubated in the same medium. At various times postinfection, a mixture of NP-40 and sodium deoxycholate is added directly to the medium on the infected monolayers to final concentrations of 1% and 0.5%, respectively. The lysates are then harvested and virus yields are determined by plaque titration on L-929 cells.

Radiolabelling of adenovirus-infected cells and preparation of lysates

Confluent monolayers of cells are infected with adenovirus (MOI ~ 10 PFU/cell). At various times postinfection, the media is replaced with methionine-free DMEM containing 10% dialyzed PBS and 0.1 mCi/ml [^{35}S]methionine. After further incubation for 1 hour at 37°C, the cells are washed in phosphate-buffered saline (PBS) and lysed in the same buffer containing 1% Triton X-100, 0.5% sodium deoxycholate and 1 mM EDTA. The nuclei are then removed by low speed centrifugation and the supernatants stored at 70°C until use.

Immunoprecipitation and SDS-PAGE analysis

Standard immunoprecipitation of [^{35}S]-labelled adenovirus-infected cell lysates with anti-adenovirus serum is done. Immunoprecipitates are analyzed by discontinuous SDS-PAGE according to the protocol of Laemmli (Laemmli, U.K., (1970) *Nature*, *227*:680-685).

Breast Cancer

The c-*erbB-2/neu* gene encodes a transmembrane protein with extensive homology to the EGFR that is overexpressed in 20-30% of patients with breast cancer (Yu, D. *et al.* (1996) *Oncogene 13*:1359). Ras activation, either through point mutations or through augmented signaling cascade elements upstream of Ras (including the c-erbB-2/neu homologue EGFR) ultimately creates a hospitable environment for virus replication, an array of cell lines derived from human breast cancers are assayed for adenovirus susceptibility. The cell lines included MDA-MD-435SD (ATCC deposit HTB-129), MCF-7 (ATCC deposit HTB-22), T-27-D (ATCC deposit HTB-133), BT-20 (ATCC deposit HTB-19), HBL-100 (ATCC deposit HTB-124), MDA-MB-468 (ATCC deposit HTB-132), and SKBR-3 (ATCC deposit HTB-30).

Based upon induction of cytopathic effects and viral protein synthesis as measured by radioactive metabolic labeling and immunofluorescence as described above, sensitivity to infection may be determined.

Brain Glioblastoma

Human brain glioblastoma cell lines A-172, U-118, U-178, U-563, U-251, U-87 and U-373 (these cells are a generous gift from Dr. Wee Yong, University of Calgary) are tested to determine the susceptibility to adenovirus infection.

To assess the sensitivity of these cells to adenovirus, cells are grown to 80% confluency and are then challenged with adenovirus at a multiplicity of infection (MOI) of 10. Within a period of 48 hours, widespread cytopathic effects will be seen. To demonstrate further that the lysis of these cells is due to replication of adenovirus, the cells are then pulse-labeled with [^{35}S]methionine for three hour periods at various times post-infection and proteins are analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described above.

U-87 cells are also introduced as human tumor xenografts into the hind flank of 10 SCID mice. U-87 cells are grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described above. Cells are harvested, washed, and resuspended in sterile PBS; 2.0 x 10^6 cells in 100 μl, and are injected subcutaneously at a site overlying the hind flank in five- to eight-week old male SCID mice (Charles River, Canada). Tumor growth is measured twice weekly for a period of four weeks.

To determine if there is viral spread beyond the tumor mass, immunofluorescent microscopy using antibodies directed against total adenovirus

proteins is conducted, as described above, on paraffin sections of the tumor and adjoining tissue.

Since most tumors are highly vascularized, it is likely that some virus may enter the blood stream following the lysis of the infected tumor cells. To determine if there is systemic spread of the virus, blood is harvested from the treated and control animals, serially diluted for subsequent plaque titration, and the concentration of infectious virus in the blood is determined.

The high degree of tumor specificity of the virus, combined with systemic spread, suggest that adenovirus can replicate in glioblastoma tumors remote from the initially infected tumor. SCID mice are implanted bilaterally with U-87 human tumor xenografts on sites overlying each hind flank of the animals. These tumors are allowed to grow until they measure 0.5 x 0.5 cm. The left-side tumors are then injected with a single dose (1×10^7 pfu) of adenovirus in treated animals (n=5); control animals (n=7) are mock-treated with UV-inactivated virus. Tumors are again measured twice weekly for a period of four weeks.

Pancreatic Carcinoma

Cell lines derived from pancreatic cancer are investigated for their susceptibility to adenovirus infection, using processes described above. The cell lines included Capan-1 (ATCC deposit HTB-79), BxPC3 (ATCC deposit CRL-1687), MIAPACA-2 (ATCC deposit CRL-1420), PANC-1 (ATCC deposit CRL-1469), AsPC-1 (ATCC deposit CRL-1682) and Hs766T (ATCC deposit HTB-134).

The assays described above may be modified by one skilled in the art to test the susceptibility of cells to other types of virus, such as HSV, vaccinia virus and parapoxvirus orf virus.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

What is claimed is:

1. A method for treating a Ras-mediated cell proliferative disorder in a mammal, comprising administering to proliferating cells in a mammal having a Ras-activated pathway an effective amount of one or more viruses selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells.

2. The method of Claim 1, wherein the modified adenovirus lacks the gene encoding VAI RNA.

3. The method of Claim 1, wherein the modified HSV comprises a HSV having a mutation in the $_{\gamma 1}$34.5 gene.

4. The method of Claim 1, wherein the modified vaccinia virus comprises a mutant gene selected from the group consisting of E3L and K3L.

5. The method of Claim 4 wherein the mutant gene is E3L.

6. The method of Claim 4 wherein the mutant gene is K3L.

7. The method of Claim 1 wherein the parapoxvirus orf virus comprises a parapoxvirus orf virus having a mutation in the OV20.0L gene.

8. The method of Claim 1, wherein more than one type of virus is administered.

9. The method of Claim 1, wherein more than two strains of virus are administered.

10. The method of Claim 1, wherein the Ras-mediated proliferative disorder is a neoplasm.

11. The method of Claim 1, wherein the Ras-mediated proliferative disorder is neurofibromatosis.

12. The method of Claim 10, wherein the neoplasm is a solid neoplasm.

13. The method of Claim 10, wherein the neoplasm is selected from the group consisting of lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer and central and peripheral nervous system cancer.

14. The method of Claim 13, wherein the neoplasm is a central nervous system cancer.

15. The method of Claim 13, wherein the neoplasm is breast cancer.

16. The method of Claim 12, wherein the neoplasm is a hematopoietic neoplasm.

17. The method of Claim 12, wherein the virus is administered by injection into or near the solid neoplasm.

18. The method of Claim 1, wherein the virus is administered intravenously into the mammal.

19. The method of Claim 1, wherein the virus is administered intraperitoneally into the mammal.

20. The method of Claim 1 wherein the mammal is immunocompetent.

21. The method of Claim 20 wherein the virus is immunoprotected.

22. The method of Claim 21 wherein the virus is encapsulated in a micelle.

23. The method of Claim 20 wherein the virus is administered with an effective amount of an anti-antivirus antibody.

24. The method of Claim 1, wherein the mammal is a human.

25. The method of Claim 1, wherein approximately 1 to 10^{15} plaque forming units of virus/kg body weight are administered.

26. The method of Claim 1, wherein the virus is administered in a single dose.

27. The method of Claim 1, wherein the virus is administered in more than one dose.

28. The method of Claim 10, wherein the neoplasm is metastatic.

29. The method of Claim 1 further comprising the administration of an effective amount of a chemotherapeutic agent.

30. The method of Claim 10, wherein the virus is treated with a protease prior to administration.

31. A method of treating a neoplasm having an activated Ras-pathway in a human, comprising administering to the neoplasm an effective amount of virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, to result in substantial oncolysis of the neoplasm.

32. The method of Claim 31, wherein the neoplasm is a solid neoplasm and the virus is administered by injection into or near the neoplasm.

33. The method of Claim 32, wherein the solid neoplasm is pancreatic cancer.

34. A method of inhibiting metastasis of a neoplasm having an activated Ras-pathway in a mammal, comprising administering to the neoplastic cells in a mammal a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, in an amount sufficient to result in substantial lysis of the neoplasm.

35. The method of Claim 34 wherein the mammal is selected from the group consisting of dogs, cats, sheep, goats, cattle, horses, pigs, humans and non-human primates.

36. A method of treating a neoplasm suspected of having an activated Ras-pathway in a mammal, comprising surgical removal of the substantially all of the neoplasm and administration of a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified

parapoxvirus orf virus, to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplasm.

37. A pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, a chemotherapeutic agent and a pharmaceutically acceptable excipient.

38. A pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a pharmaceutically acceptable excipient.

39. The pharmaceutical composition of Claim 38 further comprising an immunoprotected virus.

40. A kit comprising a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and a chemotherapeutic agent.

41. A kit comprising a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus and an anti-antivirus antibody.

42. A method for treating a population of cells comprising a neoplasm suspected of having an activated Ras-pathway *in vitro* comprising administering to said population of cells *in vitro* a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified

parapoxvirus orf virus in an amount sufficient to result in substantial lysis of the neoplasm.

43. A method for treating a Ras-mediated proliferative disorder in a mammal, comprising the steps of:

a) performing a step selected from the group consisting of:

i) administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent;

ii) removing B-cells or T-cells from said mammal;

iii) removing anti-virus antibodies from said mammal;

iv) removing antibodies from said mammal;

v) administering anti-antivirus antibodies to said mammal; and

vi) suppressing the immune system of the mammal; and

b) administering to the proliferating cells in said mammal an effective amount of one or more viruses selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus under conditions which result in substantial lysis of the proliferating cells;

wherein the anti-virus antibodies are selected from the group consisting of anti-adenovirus antibodies, anti-HSV antibodies, anti-vaccinia virus antibodies and anti-parapoxvirus orf virus antibodies and wherein the anti-antivirus antibodies are selected from the group consisting of anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies.

44. The method of Claim 43, wherein the Ras-mediated proliferative disorder is a neoplasm.

45. The method of Claim 43, wherein the Ras-mediated proliferative disorder is neurofibromatosis.

46. The method of Claim 44, wherein the neoplasm is a solid neoplasm.

47. The method of Claim 44, wherein the neoplasm is selected from the group consisting of lung cancer, prostate cancer, colorectal cancer, thyroid cancer, renal cancer, adrenal cancer, liver cancer, pancreatic cancer, breast cancer and central and peripheral nervous system cancer.

48. The method of Claim 47, wherein the neoplasm is a central nervous system cancer.

49. The method of Claim 47, wherein the neoplasm is breast cancer.

50. The method of Claim 44, wherein the neoplasm is a hematopoietic neoplasm.

51. The method of Claim 46, wherein the virus is administered by injection into or near the solid neoplasm.

52. The method of Claim 43, wherein the virus is administered intravenously into the mammal.

53. The method of Claim 43, wherein the virus is administered intraperitoneally into the mammal.

54. The method of Claim 43 wherein the mammal is immunocompetent.

55. The method of Claim 54 wherein the virus is immunoprotected.

56. The method of Claim 43 wherein the virus is encapsulated in a micelle.

57. The method of Claim 43 wherein prior to step (a) an effective amount of one or more viruses is administered under conditions which result in substantial lysis of the proliferating cells.

58. The method of Claim 43, wherein the mammal is a human.

59. The method of Claim 43, wherein approximately 1 to 10^{15} plaque forming units of virus/kg body weight are administered.

60. The method of Claim 43, wherein the virus is administered in a single dose.

61. The method of Claim 43, wherein the virus is administered in more than one dose.

62. The method of Claim 44, wherein the neoplasm is metastatic.

63. The method of Claim 43 further comprising the administration of an effective amount of a chemotherapeutic agent.

64. The method of Claim 43, wherein the virus is treated with a protease prior to administration.

65. The method of Claim 44, wherein the neoplasm is pancreatic cancer.

66. The method of Claim 44 further comprising surgical removal of the substantially all of the neoplasm and administration of the virus to the surgical site in an amount sufficient to result in substantial oncolysis of any remaining neoplastic cells.

67. The method of Claim 43, wherein step a) is administering to the proliferating cells in said mammal an effective amount of an immune suppressive agent.

68. The method of Claim 67 wherein the immune suppressive agent is administered concurrently with the virus.

69. The method of Claim 67 wherein the immune suppressive agent is administered prior to the virus.

70. The method of Claim 67 wherein the immune suppressive agent is selected from the group consisting of cyclosporin, rapamycin, tacrolimus, mycophenolic acid, azathioprine, analogs of the above agents, radiation, HIV infection and anti-antivirus antibodies.

71. A pharmaceutical composition comprising an immune suppressive agent, a virus, and a pharmaceutically acceptable excipient.

72. The pharmaceutical composition of Claim 71 further comprising a chemotherapeutic agent.

73. The pharmaceutical composition of Claim 71 comprising at least one selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus, and at least one immunoprotected virus selected from the group consisting of an immunoprotected modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

74. A kit comprising a pharmaceutical composition comprising an immune suppressive agent and a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

75. The kit of Claim 74 further comprising a chemotherapeutic agent.

76. The method of Claim 43, wherein step a) is removing B-cells or T-cells from said mammal.

77. The method of Claim 76 wherein the B-cells or T-cells are removed by filtration and heme-dialysis.

78. The method of Claim 77 wherein the filtration is conducted with affinity chromatography, wherein the affinity chromatography comprises antibodies immobilized to a solid support.

79. The method of claim 78 wherein the antibodies are anti-T-cell or anti-B-cell antibodies.

80. The method of Claim 76 wherein the B-cells and T-cells are removed by radiation therapy.

81. The method of Claim 76 wherein the B-cells and T-cells are removed by the use of cyclic steroids.

82. The method of Claim 81 wherein the cyclic steroid is cyclosporin.

83. A kit comprising: a) a means for removing B-cells or T-cells from a mammal; and b) a pharmaceutical composition comprising a virus selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

84. The kit of Claim 83 further comprising a chemotherapeutic agent.

85. The method of Claim 43, wherein step a) is removing anti-virus antibodies from said mammal.

86. The method of Claim 85 wherein the anti-virus antibodies are removed by heme-dialysis and passing the blood over immobilized virus.

87. The method of Claim 86 wherein the virus is affixed to a solid support.

88. A kit comprising:

a) a means for removing anti-virus antibodies from said mammal; and

b) a pharmaceutical composition comprising a virus;

wherein the anti-virus antibodies are selected from the group consisting of wherein the anti-virus antibodies are selected from the group consisting of anti-adenovirus antibodies, anti-HSV antibodies, anti-vaccinia virus antibodies and anti-parapoxvirus orf virus antibodies and the virus is

selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

89. The kit of Claim 88 further comprising a chemotherapeutic agent.

90. The method of Claim 43, wherein step a) is removing antibodies from said mammal.

91. The method of Claim 90 wherein said antibodies are IgG antibodies.

92. The method of Claim 90 wherein the antibodies are removed by heme-dialysis and passing the blood over immobilized protein A.

93. The method of Claim 43, wherein step a) is administering anti-antivirus antibodies to the mammal.

94. The method of Claim 93 wherein the anti-antivirus antibodies are administered intravenously.

95. The method of Claim 93 wherein the anti-antivirus antibodies are humanized antibodies.

96. A pharmaceutical composition comprising an anti-antivirus antibody, a virus, and a pharmaceutically acceptable excipient, wherein the anti-antivirus antibodies are selected from the group consisting of anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies and the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

97. The pharmaceutical composition of Claim 96 further comprising a chemotherapeutic agent.

98. The pharmaceutical composition of Claim 96 comprising at least one selected from the group consisting of virus and an immunoprotected virus.

99. A kit comprising a pharmaceutical composition comprising an anti-antivirus antibody and a virus;

wherein the anti-antivirus antibodies are selected from the group consisting of anti-antiadenovirus antibodies, anti-antiHSV antibodies, anti-antivaccinia virus antibodies and anti-antiparapoxvirus orf virus antibodies and the virus is selected from the group consisting of modified adenovirus, modified HSV, modified vaccinia virus and modified parapoxvirus orf virus.

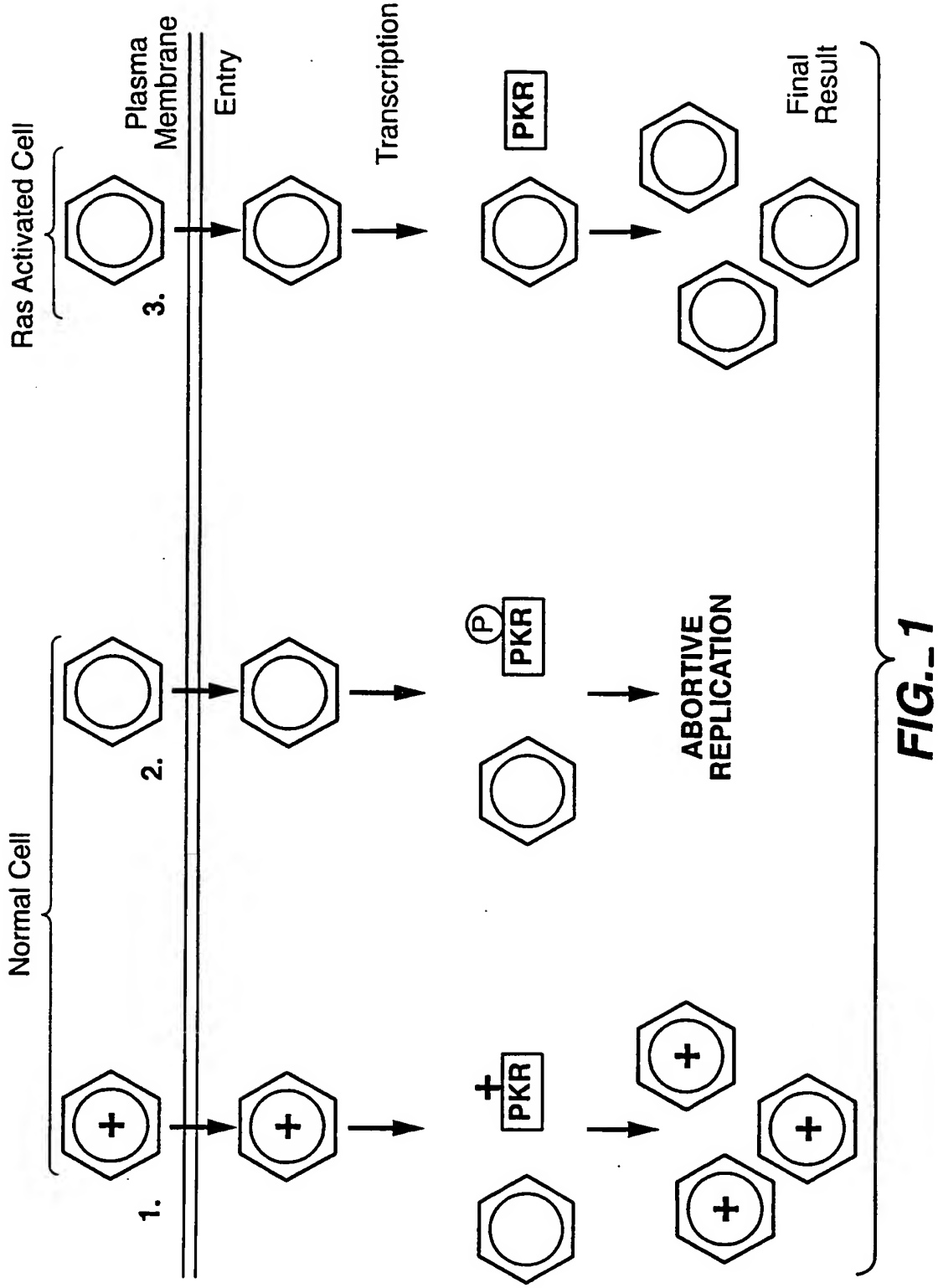
100. The kit of Claim 99 further comprising a chemotherapeutic agent.

101. The method of Claim 43, wherein step a) is suppressing the immune system of the mammal.

102. The method of Claim 101 wherein the immune system is suppressed by radiation therapy.

103. The method of Claim 101 wherein the immune system is suppressed by cyclic steroids.

104. The method of Claim 103 wherein the cyclic steroid is cyclosporin.


Ras Activated Cell
Plasma Membrane
Entry
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PKR
Final Result
3.
Normal Cell
2.
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PKR
ABORTIVE REPLICATION
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FIG._1

INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/CA 00/01329

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K35/76 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
WPI Data, PAJ, EPO-Internal, BIOSIS, MEDLINE, CANCERLIT, CHEM ABS Data

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

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Date of the actual completion of the international search	Date of mailing of the international search report
26 February 2001	14/03/2001

Name and mailing address of the ISA	Authorized officer
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